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INVESTIGATIONS ON THE VIRUS OF HERPES SIMPLEX

THE HERPES SIMPLEX COMPLEMENT FIXATION TEST AND ITS USE IN THE STUDY OF HERPES ANTIBODIES

BY

PEKKA HALONEN

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MERCATORIN KIRJAPAINO
HELSINKI, FINLAND

FROM THE DEPARTMENT OF VIROLOGY, UNIVERSITY OF HELSINKI

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To my wife

Translated by
Eva Palmgren

PREFACE

The present investigation was carried out at the Department of Virology, the University of Helsinki, during the period 1951—1954.

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P. H.

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SURVEY OF THE LITERATURE

HISTORY OF THE STUDY OF HERPES SIMPLEX

The infectious character of herpes febrilis was demonstrated in 1873 by Vidal (156). He injected fluid from a herpetic vesicle into the skin of the host, and obtained a similar lesion at the site of injection. The fluid of the latter was likewise capable of producing vesicles. Experiments in which the infectious agent of the herpes vesicle was successfully transferred to experimental animals were first described in 1919 by Löwenstein (100, 101). Grüter, however, stated that in 1913 he had transferred herpetic keratitis onto the cornea of a rabbit, though his results were not published until 1920 (73, 74). Even in his first investigations of the virus of herpes simplex, Löwenstein disclosed many of its characteristic features. He observed that this virus can be transferred from herpetic vesicles onto the cornea of the rabbit, where it produces typical herpetic keratoconjunctivitis. A similar effect was not obtained with the fluid from herpes zoster vesicles. Furthermore Löwenstein showed that the virus of herpes simplex is easily destroyed by heat, maintains its potency during passage through many rabbit corneas, produces »bodies» in the affected tissues and with difficulty passes through Berkefeld filters.

In 1920 Doerr (42) observed that herpes infection in rabbits occasionally extended to the brain, causing encephalitis. This affinity of the herpes virus for the brain was the main concern of a large number of investigations on herpes simplex published in the nineteen-twenties. It was believed that the causative factor in encephalitis epidemica had been traced. This view found support when Levaditi and Harvier (95) succeeded in isolating a strongly neurotropic strain of herpes from the brain tissue of a patient who had died of this disease. Later this virus was also isolated from brain tissue in several other cases of fatal encephalitis, but, on the other hand, there were many workers who did not succeed in isolating herpes virus in this disease in spite of investigating fairly large series (38, 39, 54, 166). The serological investigations of Andrewes and Carmichael (10) also constituted evidence against Levaditi's theory regarding the herpes virus as the cause of encephalitis epidemica. The role of the virus of herpes simplex in the etiology of this disease is not yet fully understood. It has been the established cause of some fatal cases of encephalitis, but these seem to have

been exceptional, and often they have displayed a clinical picture differing from encephalitis epidemica (von Economo's disease).

In 1921 Blanc and Caminopetros (23) showed that, in addition to the rabbit and the guinea-pig, the mouse can also be used in the investigation of herpes. The mouse has proved to be a very satisfactory experimental animal where accurate determinations of antibody content are involved. In 1929, Gildemeister *et al.* (67) cultivated herpes in tissue cultures. In the nineteen-thirties the use of fertile hen's eggs was adopted in virological research; their usefulness in the cultivation of herpes virus was demonstrated by Goodpasture *et al.* (68a) and by Saddington (128). After one or two days' growth the virus was found to produce typical pock lesions on the chorioallantoic membranes of the eggs. When it was inoculated into the amniotic, allantoic or yolk sac, or directly into the embryo, the latter died within 2 to 4 days, depending on the strain. The extra-embryonic fluids of eggs infected in this way and suitably incubated contained 10^4 to 10^7 living particles (20). Today the virus of herpes simplex is mostly isolated on the chorioallantoic membrane of the embryonated egg. This also constitutes suitable material for the neutralization test (33). The extra-embryonic fluids are employed as antigen in the complement fixation and dermal reaction tests. Thus the embryonated egg has become one of the most widely used and helpful tools in the study of the virus of herpes simplex.

To begin with, the mechanism of herpetic infection seemed confusing, since it was found that herpes vesicles are only encountered in subjects who have neutralizing antibodies to herpes (10). It was a well-known fact that virus diseases are generally transmitted to individuals who lack the specific antibody. Hence it seemed doubtful whether herpes really was an infectious disease. Burnet's theory regarding the »natural history» of the herpes virus represents the current view on the infective mechanism of herpes simplex and the behaviour of this virus in its natural host, *i.e.* man. In a series of serological investigations (32, 33, 34, 35, 36) Burnet showed that children seldom have herpes antibodies; he found that the development of these is, as a rule, associated with stomatitis, and that herpes virus may then be isolated from the mouth of the patient. He stated that primary herpes simplex infection in childhood often produces severe systemic illness with high fever, usually taking the form of stomatitis. Furthermore he assumed that those who have suffered from a primary herpes infection become carriers of herpes virus, which subsequently remains mostly latent, but under the influence of various stimuli may become active and produce vesicles. Later it has been found that a primary infection may involve the genitals, the skin of the face and body, and the eyes. The occurrence of a primary herpes infection has been serologically demonstrated even in the absence of any clinical evidence (8, 112).

Different strains of herpes simplex, isolated in different clinical entities, have proved to be antigenically largely similar. The principal difference observed is that some strains display a tendency to affect the central nervous system of experimental animals and others to produce cutaneous signs. Hence it was suggested that some strains of herpes simplex are

neurotropic and others dermotropic. Later is has been demonstrated, however, that the difference is often inherent in the host animal rather than in the strain of virus. Thus a strain that has caused fatal encephalitis in man may produce chiefly cutaneous signs in the rabbit and slight or no symptoms from the central nervous system (57). Nonetheless, serological differences between different strains of herpes simplex have been demonstrated both by the neutralization and the complement fixation test (57, 143, 162).

On microscopical investigation of tissues infected with herpes virus, various kinds of »bodies» have been found, eosinophilic intranuclear inclution bodies being the most significant. Today these are regarded as very typical of the virus of herpes simplex, although Lipschütz, who in 1921 was the first to describe them (97, 98), stated that he had seen similar intranuclear bodies in the cornea of a rabbit infected with the fluid from herpes zoster vesicles. It is now believed that the intranuclear inclusions consist of living virus and are not cellular degeneration products due to the infection, as has also been suggested. They are round or oval and generally occupy the whole of the nucleus. Intranuclear inclusions have diagnostic significance, inasmuch as their presence constitutes histological confirmation, for instance in cases of fatal encephalitis, that infection has been due to the virus of herpes simplex.

In the last few years a large number of investigations on the virus of herpes simplex have been published, the main purpose of which has been not so much to throw light on this agent and the diseases possibly due to it, as to gain further insight into the nature of viruses (1, 2, 6, 14, 21, 37, 60, 107, 108, 109, 121, 137, 148, 161).

DISEASES CAUSED BY THE VIRUS OF HERPES SIMPLEX

It is a feature common to the various conditions caused by the herpes virus, that in primary infection there is usually a severe systemic illness in addition to a local lesion, whereas, in the recurrent forms, the general reaction is mild, even though local lesions may be severe. In cases of primary infection, where no antibody to herpes is present in the acute stage, the patient usually has high fever and looks very ill. Prognosis is mostly favourable, however, although many fatal cases are mentioned in the literature.

Herpes Simplex and Herpes Febrilis.[1] — These names are mostly used for vesicles occurring on the lips or on the face in association with or independently of fever. Initially a reddish papule appears on the skin or the mucous membrane, which in a few hours becomes filled with transparent fluid. This contains herpes virus in abundance. When the vesicle has ruptured, a crust is formed, which heals in some days without leaving any scar. In herpetic individuals such vesicles develop in connection with

[1] Principally the classification suggested by van Rooyen and Rhodes (154) and Scott (134) has been followed in denominating the diseases.

various stimuli such as a cold, diseases accompanied by high fever, menstruation, emotional upsets, artificial fever therapy, gastric disorders, *etc.* In subjects with this disposition vesicles may even be induced under hypnosis (78, 153).

Herpes Genitalis. — Herpes vesicles occasionally occur on both the male and the female genitals, and they may be propagated by sexual intercourse (12, 80, 141). In primary infection herpes virus may also cause vulvovaginitis and urethritis (46, 93, 142).

Eczema Herpeticum. — In 1887 Kaposi (86) described a varicelliform eruption, sometimes occurring in infantile eczema patients as a grave complication. This disease has been referred both to bacterial and to virus infection. From some patients vaccinia virus has been isolated, or there has been a history of recent exposure to vaccinia virus. In 1941 Seidenberg (140) isolated herpes simplex virus from the vesicles of a patient with Kaposi's varicelliform eruption. Later a large number of papers have been published in which it has been shown, either by means of isolation of virus or serologically, that all cases of Kaposi's eruption in the series concerned, orsome of them, have been due to herpes virus (15, 28, 49, 52, 72, 89, 103, 125, 146, 157). For cases of Kaposi's varicelliform eruption caused by herpes virus the name eczema herpeticum has been adopted. Not all cases of eczema herpeticum have been primary herpes infections, antibody to herpes having been found even in the acute stage of the disease in some patients from whom herpes virus has been isolated (47). In adults, Boake, Dudgeon and Burnet (26) and Pette (117) observed recurrent eruptions, due to herpes virus and resembling Kaposi's varicelliform eruption.

Herpes Stomatitis. — The acute, infectious gingivostomatitis of children is generally regarded as a primary infection with the virus of herpes simplex. It is characterized by systemic illness with high fever and severe inflammation of the mouth. In its gravest forms it is also accompanied by vesicles elsewhere on the mucous membranes and the skin. In many cases of stomatitis, and according to certain investigators in all, antibody to herpes develops during the course of the illness, and then herpes virus may be isolated from the patient's mouth (8, 22, 31, 34, 35, 41, 61, 69, 75, 99, 136). In 1949 Rogers *et al.* (122) showed that in adults acute gingivostomatitis may also be a primary herpes simplex infection with the same clinical picture as in children. The recurrent form of stomatitis has also been referred to herpetic infection (87, 136, 154), but according to Dodd and Ruchman (40) and Blank *et al.* (25) this agent is not the causative factor in recurrent stomatitis and recurrent aphthous ulcers. Scott (134) regards recurrent herpes stomatitis as a possibility, though he believes it to be extremely rare.

Diseases of the central nervous system due to the virus of herpes simplex. — In 1941 Smith, Lennette and Reames (145) isolated herpes virus from the brain tissue of a child who had died of encephalitis; on histological examination intranuclear inclusion bodies were found in the cerebral lesions. These writers also surveyed the records on encephalitis in which virus had been

isolated; the virus had definitely been identified as that of herpes in some 10 cases. Encephalitis in which herpes virus has been isolated has subsequently been described by Fisher and Patrick in 1947 (51), Fastier and Alexander in 1950 (48), Paillard, Wildi and Wirth in 1950 (113), Quilligan and Wilson in 1951 (120), Draheim and De Rodaniche in 1952 (43), Florman and Mindlin in 1952 (56), and by Tongeren and Jong in 1952 (155).

Furthermore cases of encephalitis where herpes virus had been isolated and, in addition, intranuclear inclusion bodies had been found, were reported in 1944 by Zarafonetis *et al.* (165), in 1946 by Whitman, Wall and Warren (159), whose series included two adults, and in 1951 by Ginder and Whorton (68). In 1951 Wildi (160) isolated herpes virus from the brain tissue of a child who had died of encephalitis, and demonstrated histologically the presence of inclusions, which he did not, however, regard as typical of herpes.

The presence of intranuclear inclusion bodies in encephalitis was reported by Dawson (38, 38a), Akelaitis (5), Kinney (88), Swan (149), Malamud, Haymaker and Pinkerton (104), Greenfield (71), and France and Wilmers (59).

An increase in herpes antibodies in patients with encephalitis, meningoencephalitis or aseptic meningitis was reported in 1951 by Afzelius-Alm (4), in 1952 by Scott *et al.* (138), in 1953 by Adair, Gauld and Smadel, (3) and in 1955 by Penttinen *et al.* (115).

In 1943 Armstrong (11) isolated herpes virus from the cerebrospinal fluid of a patient displaying mild symptoms of choriomeningitis. He assumed that herpes virus is in rare instances the cause of lymphocytic meningitis. In 1942 Janbon, Chaptal and Labraque-Bordenave (82) also isolated herpes virus from the cerebrospinal fluid of a patient with meningitis.

Furthermore it has been shown in a number of papers that the occurrence of herpes vesicles is associated with various symptoms of the central nervous system. Mention may be made of the case described in 1929 by Pette (117), where a patient displayed recurrent signs of acute meningitis invariably accompanied by severe herpes facialis over the whole of the face.

According to Braley (29) the virus of herpes simplex is one of the causative factors in *acute keratoconjunctivitis*, *dendritic ulcers*, *keratitis disciformis* and *chronic keratitis bullosa*.

In 1952 six cases of *herpetic oesophagitis* were reported by Fingerland, Vortel and Endrys (50), who concluded that herpetic ulcers of the oesophagus are perhaps commoner than has been assumed. In 1950 Ruchman and Dodd (126) described *herpetic rhinitis*. *Infantile herpetic hepatitis* was recorded by Quilligan and Wilson (120), by Zuelser and Stulberg (168) and by McDougal *et al.* (105), in association with fatal herpetic infection. In certain cases of *erythema multiforme* the herpes virus has been credited with some etiological significance (7, 90, 106, 123, 163).

Neutralization Tests

In the first neutralization tests with herpes simplex, the infectivity of a serum-virus mixture was determined on the cornea or in the brain of the rabbit (53, 96, 102). To begin with conflicting, or even negative, results were obtained regarding the presence of herpes antibody in the sera of rabbits and human subjects that had recovered from herpetic infection (24, 116). Of the human sera examined along these lines by Levaditi, some showed a neutralizing capacity, whilst others even increased the infectivity of the serum-virus mixture under investigation as compared with the controls. In 1925 Flexner and Amoss (55) described a neutralization test on rabbit brain yielding consistent results. They mixed the sera of rabbits that had recovered from herpetic infection with a virus suspension obtained from infected rabbit brains; the mixture was incubated for half an hour at 37°C and overnight at 4°C. Thereafter it was injected intracerebrally into rabbits, and the neutralizing capacity of the sera was estimated from the death rate of the animals. It was found that rabbit immune-serum had a neutralizing effect on both the homologous strain and two other herpes strains. The same technique was also employed in testing human sera, some of which were found to contain neutralizing antibodies.

The intracerebral neutralization test in the rabbit was further elaborated by Zinsser and Tang (166), who observed that the virulence of herpes virus is rapidly lost on incubation in saline, and that such a change does not occur in the presence of normal serum. Thus they were able to explain why the above-mentioned workers had noted increased infectivity titres in neutralization tests with certain sera. In their experiments Zinsser and Tang employed 10 lethal doses of herpes virus and left the virus-serum mixture for 5 to 6 hours at 37.5°C. By this technique they found that neutralizing antibodies develop in rabbit serum only when it is immunized with living virus. In 1929 (167), using the same method, these workers performed the first extensive study ou herpes antibody in normal human sera. In their material antibodies were present in 59 per cent of the sera. Bedson and Crawford (19) measured the infectivity of a serum-virus mixture in the skin of the guinea-pig, where infective herpes virus produced vesicles. By

this technique they found that the sera of guinea-pigs recovered from herpetic infection contained neutralizing herpes antibodies by the seventh to tenth day following inoculation; furthermore they found that it was possible to increase the neutralizing activity of the serum by hyper-immunizing the rabbit. The same method was employed in Bedson's investigation of 1928 (17) on the »viricidal action» of guinea-pig serum immunized with herpes virus. The intracerebral neutralization technique in rabbits was also used by Schultz and Hoyt (130) for demonstrating the presence of antibodies in immune rabbit serum. In the same paper they surveyed in detail the earlier literature on neutralization tests with herpes.

Gay and Holden (62) recommended the use of rabbit or guinea-pig skin for determinations of the infectivity of virus-serum mixtures, claiming that this method is more economical than the intracerebral neutralization test in rabbits and gives equally good results. The former technique was also used by Andrewes and Carmichael, who tested postencephalitic and other human sera for herpes antibody.

In 1932 Weyer (158) used the mouse in neutralization tests. An emulsion containing 5 per cent herpes-infected mouse brain was added to the undiluted serum under investigation; the mixture was left at room temperature for about one hour and was subsequently inoculated intracranially into two mice. If the serum did not contain neutralizing herpes antibody, the animals died within 5 to 10 days. By this method the incidence of herpes antibody in different age groups was determined. Using the same technique, Hudson, Cook and Adair (81) investigated the relationship between herpes antibody and sex, pregnancy and menstruation.

Gildemeister and Ahlfeld (65) employed a neutralization technique with herpes involving subcutaneous injection of the serum under investigation into mice, followed in a few minutes by intracutaneous injection of herpes virus. Their percentage figures on the incidence of herpes antibody in normal human sera are lower than those obtained by other methods.

In 1941 Scott, Steigman and Convey (136) elaborated a neutralization test in mice yielding reliable herpes antibody titres. They made a series of tenfold dilutions of infected mouse brain in broth. The undiluted serum under investigation was added, and after incubation at room temperature for one hour and for 3 hours in the

ice-box, each of the virus-serum mixtures was injected into four mice. The antibody titre of the serum was calculated from the death rate of the mice by Reed-Muench's method. In this way the development of herpes antibody in patients with stomatitis was studied during the course of the illness. The same method was employed by Black in 1942 (22), and by Armstrong in 1944 (11). The current modification of the neutralization test in mice was elaborated by Ruchman, Welsh and Dodd in 1947 (127) and by Ruchman and Dodd in 1948 (125).

In 1939 Burnet and Lush (33) described a neutralization test carried out in the embryonated hen's egg. They inoculated the chorioallantoic membranes of 12-day eggs with a serum-virus mixture and counted the pocks developing on the membranes after 2 to 3 days' incubation; the level of neutralizing herpes antibody was estimated from the pock count. Giving satisfactory results this neutralization test was much simpler than previous methods. When Burnet tested human sera for the presence of herpes antibody using this technique, he found that all positive sera caused an almost equal decrease in the pock count on the chorioallantoic membrane. Hence he assumed that herpes antibodies are either totally lacking, or are present at a constant titre, an »all or none» phenomenon being thus involved. The neutralization test on the chorioallantoic membrane has become one of the most widely used aids in the determination of herpes antibody (4, 28, 31, 72, 77, 79, 119, 120, 124).

Another neutralization test in embryonated hen's eggs involves injection of the virus-serum mixture into the yolk sac and calculation of antibody titres from the death rate of the eggs (64, 84, 85).

Complement Fixation Tests

In the middle of the nineteen-twenties, attempts were made to use the complement fixation test as an aid in the serological investigation of the virus of herpes simplex and in the diagnosis of the clinical conditions referable to this agent. In previous work dealing with certain other viruses, for instance vaccinia, it had been found that virus antigens and antibodies may be successfully studied by means of this reaction. In 1925 Todorovitch (152) reported positive fixation, employing herpetic vesicle fluid as antigen and the serum

of monkeys immunized with herpes. In the same year Greenbaum and Harkins (70) failed to demonstrate specific fixation using phenolized, herpes-infected rabbit brain as antigen and hyper-immune rabbit serum. In 1925 and 1926 Kraus and Takaki (91, 92) reported positive complement fixation using immune rabbit sera with »cocto» antigen, prepared from herpes-infected rabbit brain, incubated for 10 days in 50 per cent glycerin, and then for half an hour at 100°C. These workers stated that they had been able to differentiate herpes, vaccinia and rabies infection from each other with the use of »cocto» antigens. By the same method Takaki, Borris and Koref in 1926 (150) demonstrated that the Levaditi strain, isolated from the brain tissue of a patient with encephalitis, was serologically different from the encephalitis japonica virus, but identical with the herpes strains isolated by Doerr and by Luger and Lauda (102). In 1927 an attempt carried out by Bedson and Crawford (19) to demonstrate complement-fixing herpes antibodies in hyper-immune guinea-pig sera was unsuccessful, although these sera had a strong neutralizing potency.

In 1928 Schultz and Hoyt (130) failed to demonstrate complement fixation using various rabbit brain antigens; they doubted the specificity of Kraus and Takaki's, and Takaki, Borris and Koref's results. In this and other papers, Schultz and his coworkers suggested that the positive results obtained in complement fixation tests with herpes and other viruses had been due to bacterial contaminants in the antigens (129, 131, 132). In 1928, using immune rabbit sera, Gildemeister and Heuer (66) were able to demonstrate complement fixation with vaccinia, but not with herpes. Negative results were also reported by Gay and Holden (62) and by Tang and Castaneda (151), who used a glycerinated rabbit brain antigen, whereas Stroian, in 1929 (147), demonstrated complement fixation with immune rabbit sera, using herpes-infected tissue extracts as antigen.

In 1929 Bedson and Bland (18) published an investigation of fundamental significance in the subsequent study of the herpes complement fixation test. Under adequate control they demonstrated specific complement fixation with the use of infected guinea-pig pads as antigen and immune guinea-pig sera. They were able to demonstrate specific fixation even in the presence of bacterially sterile antigen, which constituted evidence against Schultz's

theory that fixation is due to bacterial contaminants. Furthermore they observed that the herpes antigen and antibody require a prolonged period of fixation if satisfactory results are to be obtained. The usefulness of this technique for the investigation of herpes antibodies in human sera was substantiated in 1932 by Brain (27), who did not consider it to be as sensitive as the neutralization test, but recommended the method, nonetheless, as being more rapid and more economical with regard to experimental animals.

Myers and Chapman, in 1937 (111), used various antigens, *e.g.* suspensions of chorioallantoic membrane and extra-embryonic fluids infected with herpes. In their own opinion their results with herpes were negative, whilst there was satisfactory evidence of fixation with vaccinia and virus III. Nonetheless some of their results appear to be specific herpes titres on a level with those obtained in many later investigations using immune-sera of experimental animals. Myers and Chapman themselves did not regard even these results as significant for the reason that most of their complement fixation tests with herpes had been negative, and the titres, when some degree of fixation was demonstrable, were low and not sufficiently different in herpes-immune and control animals. The HF strain of herpes, which they employed, did not grow satisfactorily in guinea-pig pads; hence they could not use an antigen prepared according to the method of Bedson and Bland.

In 1949 Hayward (76) elaborated a herpes complement fixation test using suspensions of chorioallantoic membranes and amniotic and allantoic fluids as antigens. She inoculated chorioallantoic membranes of 12-day eggs with herpes virus; after two days' incubation the membranes were removed and ground, and 0.5 ml of saline containing 20 per cent broth was added per membrane. Thereafter the suspension was centrifuged until the supernatant was clear. Extra-embryonic fluid antigens were prepared by inoculating the yolk sac or the amniotic or allantoic sac with virus; the fluids were harvested within 24 hours after death of the embryo. With these antigens herpetic human sera were found to be positive in dilutions up to 1/128. For the purpose of investigating the properties of the complement-fixing herpes antigen Hayward separated most of the virus particles from the antigen suspension by ultra centrifugation. Thus she established that the complement-fixing antigen is chiefly due to a soluble substance, and that it is destroyed,

more or less, on incubation at 56°C for one hour. In a later study Hayward (77) tested 95 adult sera by the complement fixation technique described above, and by the chorioallantoic neutralization test. These two methods gave similar results. Hayward regarded the amniotic and allantoic fluids, which she found almost devoid of anti-complementary properties, as preferable to the chorioallantoic membrane, although the latter was almost equally good in other respects.

Dudgeon, in 1950 (44), also studied the suitability of various egg antigens in complement fixation tests with herpes. He claimed that the membrane antigen was preferable to the amniotic and allantoic fluid antigens, because the titres obtained with the former were higher, on an average, and because, in his tests, some amniotic and allantoic fluids had been anti-complementary. He prepared his membrane antigen in the same way as Hayward, but reduced the amount of extraneous protein by alternately thawing and freezing the suspension several times in a mixture of CO_2 and alcohol. The titres noted by Dudgeon in human sera were lower than Hayward's. Dudgeon showed that the titres of complement-fixing antibody in the sera of children with acute stomatitis and Kaposi's varicelliform eruption increased during the illness, thus demonstrating the usefulness of this reaction in the serological diagnosis of clinical conditions due to the virus of herpes simplex. In 1950, Fowler (58) prepared antigen by inoculating the yolk sac of 7-day eggs and harvesting the allantoic fluid after 2 days' incubation. He titrated the fluids on the chorioallantoic membrane and discarded those with titres below 10^{-5}. Using this antigen and an immune guinea-pig serum Fowler was able to demonstrate fixation. In 1951, Afzelius-Alm (4) at first used antigen prepared from the brains of mice infected with herpes; later he used chorioallantoic membrane antigen. Although he employed only one unit of complement his titres were so low, however, that he preferred the chorioallantoic neutralization test for serological diagnosis of herpetic diseases. His technique differed from the other complement fixation tests for herpes described after the publication of Bedson and Bland, inasmuch as he incubated the antigen and the serum for 45 minutes at 37°C. Fowler alone has successfully employed fixation for one hour at 37°C, but he demonstrated complement fixation only with immune guinea-pig serum.

In 1952, Gajdusek, Robbins and Robbins (61) found that different strains of herpes simplex differ in their capacity to produce potent complement-fixing antigen in the embryonated egg. With the Z strain, which they had isolated, they were able to prepare amniotic fluid antigen of high titre. They also regarded the allantoic fluid and the yolk sac material as suitable antigens. In their complement fixation tests Gajdusek, Robbins and Robbins employed the technique used by Enders and Levens (45) for mumps. The usefulness of this complement fixation test in the study of herpetic diseases was confirmed by neutralization tests and by the demonstration of an increase in the titres in a number of patients with herpetic gingivostomatitis and eczema herpeticum. The complement fixation titres were invariably elevated where the corresponding neutralization index was high. When the neutralization index was low, the presence of complement-fixing herpes antibody could not be demonstrated.

Brown, in 1953 (30), investigated the antigenic properties of the herpes simplex virus. He separated, by centrifugation, the soluble and the particulate components in the antigens of herpes virus cultivated in embryonated eggs and in guinea-pig pads. The soluble component possessed complement-fixing and dermal reactivity properties. The complement-fixing properties were rapidly destroyed by heat, whilst dermal reactivity was heat-resistant. Brown prepared antisera to the soluble antigen by absorbing the herpes antisera of hyper-immune guinea-pigs with washed virus particles. These sera maintained complement-fixing power, but lost their neutralizing capacity. Scott *et al.*, in 1953 (139), observed that no complement-fixing activity in egg-antigens, prepared by Dudgeon's method, was demonstrable until the concentration of virus had reached at least 10^7 infectious units per membrane.

Recently Womack and Hunt (162), using the complement fixation test, have studied the serological differences between six strains of herpes simplex virus. Antisera against these strains were prepared in rabbits, and antigens were prepared separately against the different strains using the amniotic fluid of embryonated eggs. With these sera and antigens cross-complement fixation tests were performed, whereby each of the antigens fixed with the homologous antiserum, but some antisera did not fix with all antigens, at least not in the lowest dilution employed, which appears to have been

1/4 judging from a table included in the paper in question. Only one antigen fixed with all antisera. From their results these investigators conclude that striking antigenic differences exist between different herpes strains.

In the diagnosis of herpetic diseases the complement fixation test has been used by Brain, Dudgeon and Philpott in 1950 (28), by Boake, Dudgeon and Burnet in 1951 (26), by Baker, Lawton and Mc Carthy in 1952 (13), by Scott *et al.* in 1953 (138), and by Adair, Gauld and Smadel in 1953 (3). In 1953 Holzel *et al.* (79) used this test when investigating the age incidence of herpes antibody.

SUMMARY ON THE LITERATURE REGARDING THE HERPES SIMPLEX COMPLEMENT FIXATION TEST AND SCOPE OF THE PRESENT INVESTIGATION

In the nineteen-twenties, when attention was first directed to the study of herpes simplex, many investigators (19, 62, 66, 70, 111, 130, 151) published results indicating that the complement fixation test could not be used as an aid in the study of herpes antibody. Contrary results were, however, described by other writers as early as 1925 and 1926 (91, 150, 152), and the first to carry out a complement fixation test with herpes under adequate control were Bedson and Bland (18). These investigators used antigen prepared from guinea-pig pads with a hyper-immune guinea-pig serum. Subsequently their technique was used in the study of herpes antibody in human sera (27). The preparation of their antigen was inconvenient and expensive on a large scale, however, and it was not until use was made of the membranes and extra-embryonic fluids of embryonated eggs (44, 58, 76), that extensive serological investigations using the complement fixation test became possible. Even then it has repeatedly been contended that, in the serological diagnosis of herpes simplex infection, this reaction is less reliable and less convenient than the neutralization test (4, 133, 135).

The present research was undertaken in 1951 because these conflicting data and opinions seemed to prompt a thorough investigation into the usefulness and reliability of the complement fixation test in the study of herpes antibody in human sera. If positive results were obtained, it also seemed to be of interest to trace the factors responsible for the failures reported by some earlier workers.

When the reaction studied had been found to provide a satisfactory index of herpes antibody, investigations were continued in order to elucidate the following questions:

What is the incidence of herpes simplex infection amongst the population in Finland?

At what age is infection generally incurred, and

which are the clinical conditions most frequently associated with herpes infection?

OWN INVESTIGATIONS

I. THE HERPES SIMPLEX COMPLEMENT FIXATION TEST

MATERIAL AND TECHNIQUE OF THE COMPLEMENT FIXATION TEST

Choice of Antigen and of Strain of Herpes Simplex to Be Used in the Preparation of Antigen

At the outset a large number of different chorioallantoic membrane, amniotic fluid, and allantoic fluid antigens were prepared using the Armstrong 1166 strain of herpes simplex virus, either egg- or mouse-adapted; the age of the eggs, the time and temperature of incubation, and the route of inoculation varied. The complement-fixing capacity of these antigens was tested on immune rabbit and guinea-pig sera and on sera from individuals with recurrent herpes vesicles. The technique was the same as that to be described later, except that fixation was allowed for one hour at 37°C instead of overnight at 4°C. The results were completely negative, and negative results were also obtained with membrane antigens prepared using the herpes strains Pritula (egg-adapted), HF (egg-adapted) and J. R. Smith (mouse-adapted).

All these strains [1] produced typical pocks on the chorioallantoic membrane. After yolk sac inoculation the embryos died within 2 to 4 days. On rabbit cornea and in guinea-pig pads reactions were weak. After intracerebral inoculation into mice, these died within 3 to 7 days.

When fixation for one hour at 37°C was exchanged for fixation overnight at 4°C, positive results were obtained with membrane antigen, prepared with the use of some of these strains and a positive human serum in dilutions up to 1/8 and 1/16.

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[1] Obtained from the Virological Laboratory of the University of Michigan, Ann Arbor, U.S.A.

On September 28, 1953, a new strain, Kivinen, was isolated from a vesicle on a patient's lip. When fixation was allowed overnight with membrane antigen prepared with the use of this strain, herpes titres up to 1/32 and 1/64 were obtained. This strain did not produce satisfactory amniotic fluid antigen (Table 1).

The Z strain of herpes simplex virus was obtained from Dr. D. Carleton Gajdusek of the Department of Virus and Rickettsia Diseases, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington D.C., U.S.A. This strain produced a high-titre antigen in the amniotic and allantoic fluids, as has previously been demonstrated by Gajdusek, Robbins and Robbins (61). The titres obtained with membrane antigen and with pooled amniotic-allantoic fluid antigen prepared with this strain are presented in Table 1. The membrane antigen, which was prepared by the technique described by Hayward, had anti-complementary properties; hence it could only be titrated when diluted at least 1/4. The amniotic-allantoic fluid antigen was prepared by a technique to be described later. It is seen in the table that in this test the

TABLE 1.

RESULTS OF HERPES COMPLEMENT FIXATION TESTS ON A KNOWN HERPES-POSITIVE AND A KNOWN HERPES-NEGATIVE HUMAN SERUM WITH DIFFERENT ANTIGENS. ANTIGENS WERE PREPARED WITH THE KIVINEN AND Z STRAINS OF THE VIRUS OF HERPES SIMPLEX. THE + AND — SIGNS ARE EXPLAINED ON PAGE 30

Antigens	Antigen dilutions	Dilutions of the known herpes-positive serum						Dilutions of the known herpes-negative serum		
		1/4	1/8	1/16	1/32	1/64	1/128	1/4	1/8	1/16
Kivinen E 13 (chorioallantoic membrane)	1/4	+	+	+	±	—	—	∓	—	—
	1/8	+	+	+	∓	—	—	—	—	—
	1/16	+	+	—	—	—	—	—	—	—
Kivinen E 12 (amniotic fluid)	1/1	±	—	—	—	—	—	—	—	—
	1/2	—	—	—	—	—	—	—	—	—
Z E 25 (chorioallantoic membrane)	1/4	+	+	+	+	±	—	—	—	—
	1/8	+	+	+	+	—	—	—	—	—
	1/16	+	+	∓	—	—	—	—	—	—
Z E 26 (amniotic and allantoic fluid)	1/1	+	+	+	+	±	—	—	—	—
	1/2	+	+	+	+	±	—	—	—	—
	1/4	+	+	+	+	∓	—	—	—	—
	1/8	+	+	—	—	—	—	—	—	—

antigen titre of the Z membrane suspension was 1/8, and that of the Z amniotic-allantoic fluid was 1/4. Consequently these antigens had to be used in dilutions of 1/4 and 1/2. Hence the average yield of antigen per one egg was 0.5 ml × 4 = 2 ml when membranes were used, and (1 ml + 2 ml) × 2 = 6 ml when extra-embryonic fluids were used.

Since the extra-embryonic fluids had no anti-complementary properties, and considering that the yield of antigen per egg was larger, extra-embryonic fluid antigen prepared with Z strain was subsequently used in the complement fixation tests throughout the investigation.

Later another strain of herpes, Koljonen, was isolated from herpes vesicles that had developed over the whole face of a patient with pneumonia. This strain also produced potent herpes antigen in the amniotic and allantoic fluids, on a level with the Z strain material.

Choice of Fixation Time

In Table 2 are shown the results of a test in which fixation for one hour at 37°C was compared with fixation for 20 hours at 4°C. The test

TABLE 2

INFLUENCE OF FIXATION TIME AND TEMPERATURE ON HERPES COMPLEMENT FIXATION TITRES. NOS. 1, 2 AND 3 WERE KNOWN HERPES-POSITIVE HUMAN SERA; NOS. 4 AND 5 WERE KNOWN HERPES-NEGATIVE HUMAN SERA. THE + AND — SIGNS ARE EXPLAINED ON PAGE 30

Serum No.	Fixation time and temperature	Serum dilutions						Serum control
		1/4	1/8	1/16	1/32	1/64	1/128	
1	1 hour 37°C	∓	—	—	—	—	—	—
	20 hours 4°C	+	+	+	+	—	—	—
2	1 hour 37°C	+	±	—	—	—	—	∓
	20 hours 4°C	+	+	+	+	+	—	—
3	1 hour 37°C	±	—	—	—	—	—	—
	20 hours 4°C	+	+	+	—	—	—	—
4	1 hour 37°C	∓	—	—	—	—	—	∓
	20 hours 4°C	—	—	—	—	—	—	—
5	1 hour 37°C	—	—	—	—	—	—	—
	20 hours 4°C	—	—	—	—	—	—	—

was performed on three known herpes-positive and two negative human sera. In the series where fixation was allowed for one hour at 37°C only one unit of complement was used, it having been found in many previous test series that even 1.26 units of complement was excessive under the experimental conditions in question. Owing to the small amount of complement, two serum controls were incompletely haemolyzed. It is seen in Table 2 that the results using the shorter time of fixation were either negative, or the titres of the herpes-positive sera were very low. When, on the other hand, the fixation time was prolonged to 20 hours, all herpes-positive sera showed definite titres in dilutions up to 1/16, 1/32 and 1/64.

In view of these results antigen and serum were in all subsequent complement fixation tests allowed to fix overnight (for 18 to 20 hours) in the ice-box at 4°C.

Preparation of antigens

Preparation of herpes antigen. — The Z strain obtained from Dr. Gajdusek was freeze-dried 22nd egg passage. It was carried through two more passages simultaneously on the chorioallantoic membrane and in the allantoic fluid. All the different lots of Z antigens were prepared using materials from the 24th to 26th passage as inoculum. In the three first lots, allantoic fluid was used as inoculum, but subsequently this was exchanged for a suspension of chorioallantoic membrane, it having been found that the inoculum remained more active on membrane than in amniotic fluid during storage at —10 to —15°C, which was the lowest temperature available.

Twelve-day chick embryos were inoculated by the chorioallantoic route. After incubation for 48 hours at 37°C the membranes were inspected, and those showing typical, confluent pocks were aseptically removed and stored at —10 to —15°C in stoppered tubes. Immediately before infecting the antigen-eggs, the membrane to be used as inoculum was thawed and ground aseptically with powdered glass. Sufficient diluting fluid (saline containing 20 per cent broth) was added to make a 10 per cent suspension, which was centrifuged at 2500 r.p.m. for 10 minutes at 5°C. The supernatant was further diluted to $^1/_{10}$, so that the final dilution of membrane was 10^{-2}. Penicillin was added to the suspension to make a final concentration of 500 units/ml, and streptomycin 100 microgr/ml. Through a drill hole 0.2 ml of inoculum was injected with a 27-gauge needle into the yolk sac of 8-day embryos. The infected eggs were incubated at 37°C until one quarter or one third of the embryos had died; this situation was encountered after 50 to 80 hours. Deaths within 24 hours after inoculation were considered as resulting from injury on injection, and such eggs were discarded. When the above-mentioned proportion of embryos had died, the amniotic

and allantoic fluids of both dead and living embryos were harvested and pooled. The yield of antigen was centrifuged at 2000 r.p.m. for 10 minutes and stored in stoppered test tubes at —10 to —15°C for not more than two months.

Each lot of antigen was titrated against a known herpes-positive serum with antigen dilutions of $^1/_1$, $^1/_2$, $^1/_4$, $^1/_8$ and $^1/_{16}$. The antigen titre of the combined amniotic-allantoic fluid antigen was mostly $^1/_4$, with variations from $^1/_2$ to $^1/_8$. The titre of amniotic fluid alone was mostly $^1/_8$, and of allantoic fluid $^1/_2$.

The total nitrogen of undiluted, amniotic-allantoic fluid antigen determined using the micro-Kjeldahl method, varied from 0.17 to 0.54 mg/ml; the protein-nitrogen varied from 0.11 to 0.30 mg/ml.

Throughout the investigation amniotic-allantoic fluid antigen was employed either undiluted or diluted $^1/_2$.

Preparation of control antigen. — Normal amniotic-allantoic fluid of 11-day eggs was used undiluted. Three lots of control antigen were prepared. The total nitrogen of one was determined and found to be 0.38 mg/ml.

Reagents

Dilution fluid. — Saline containing magnesium and calcium chloride was adjusted with a veronal buffer to a pH of 7.4.

Cells. — Sheep cells, washed three or four times in saline and made up to 2.5 per cent in the dilution fluid, were used in all titrations.

Haemolytic amboceptor. — An amboceptor prepared for Wassermann tests was employed. It was titrated with complement dilution $^1/_{15}$, in a dilution series of 1/1000, 1/2000, 1/3000, 1/4000, 1/6000, 1/8000 and 1/10000. The smallest amount of amboceptor causing complete haemolysis was chosen as unit. Two units of amboceptor were used in the complement titration and in the test proper.

Complement. — Pooled guinea-pig serum, about 20 hours old, was used as complement. It was titrated in the presence of antigen. The complement was diluted from $^1/_{10}$ using a dilution factor of 1.26. Thus the dilution series was $^1/_{10}$, $^1/_{12{\cdot}6}$, $^1/_{15{\cdot}8}$, $^1/_{20}$, $^1/_{25}$, $^1/_{31{\cdot}6}$, $^1/_{40}$, $^1/_{50}$ and $^1/_{63}$ (114). When sensitized cells had been added to the tubes containing complement dilution and antigen, these were incubated for 45 minutes at 37°C. Immediately afterwards the degree of haemolysis was noted. The smallest amount giving complete haemolysis was taken as the unit of complement. 1.26 units of complement were used in the test proper, and the amounts indicated in Table 4 were used in the controls.

Sera. — The sera were stored at —10 to —15°C. Immediately before the tests they were inactivated for 30 minutes at 56°C. Those obtained from the Virus Department of the State Serum Institute had been inactivated in the same way before being stored at —5°C, and were inactivated again for 10 minutes at 56°C. Twofold dilutions of the sera were prepared beginning from $^1/_4$. This initial dilution was chosen owing to the fact that titration was then possible with fairly small amounts of serum, and because,

TABLE 3

RESULTS OF COMPLEMENT FIXATION TESTS ON 25 UNSELECTED HUMAN SERA, STORED FOR AT LEAST 6 MONTHS AT —10° TO —15°C. THE + AND — SIGNS ARE EXPLAINED ON PAGE 30

Group	Serum No.	Serum dilutions				Serum control dilutions	
		1/1	1/2	1/4	1/8	1/1	1/4
A	1	—	—	—	—	—	—
	2	—	—	—	—	—	—
	3	—	—	—	—	—	—
	4	—	—	—	—	—	—
	5	—	—	—	—	—	—
	6	—	—	—	—	—	—
	7	—	—	—	—	—	—
	8	—	—	—	—	—	—
	9	—	—	—	—	—	—
	10	—	—	—	—	—	—
B	11	+	+	+	+	—	—
	12	+	+	+	+	—	—
	13	+	+	+	+	—	—
C	14	+	+	+	+	±	—
	15	+	+	+	+	+	—
	16	+	+	+	±	+	—
	17	+	+	+	+	+	—
D	18	+	—	—	—	+	—
	19	+	—	—	—	+	—
	20	±	—	—	—	±	—
	21	±	—	—	—	±	—
	22	±	∓	—	—	±	—
E	23	+	±	—	—	±	—
	24	+	±	—	—	+	—
F	25	±	±	±	—	±	±

as appears in the test presented in Table 3, many sera stored for some months became anti-complementary; in the dilution ¼ this property was of no consequence. The 25 sera used in the test presented in Table 3 were an unselected series of specimens submitted for Wassermann determination; they had been stored for at least six months. Whenever fixation was demonstrable in one of these only in the dilutions 1/1 or ½, fixation was also demonstrable in the serum control diluted 1/1 (groups D and E). In all the sera which could be regarded as positive, antibody titres were higher than ¼ (groups B and C). The serum no. 25 alone was anti-complementary up to the dilution ¼.

TABLE 4

CONTROLS IN COMPLEMENT FIXATION TEST

Control tube No.	Complement			Herpes antigen	Control antigen	Unknown serum	Control positive serum	Control negative serum	Haemolytic system
	1.26 × unit	1 × unit	1/1.26 × unit						
1	×								×
2		×							×
3			×						×
4	×			×					×
5		×		×					×
6			×	×					×
7	×				×				×
8		×			×				×
9			×		×				×
10	×					×			×
11				×					×
12					×				×
13	×			×			×		×
14	×			×				×	×
15	×				×	×			×
16	×				×		×		×

The Test Proper

Unit volumes of 0.2 ml were used throughout the test, the final volume in each tube totalling 1.0 ml.

Into a series of Kahn tubes 0.2 ml each of serum dilution, antigen and complement dilution were pipetted; the mixtures were carefully shaken and kept overnight in the ice-box. The following morning, 15 minutes prior to adding the haemolytic system, equal amounts of cells and of amboceptor, diluted the day before, were mixed. To each tube 0.4 ml of sensitized cells was added, whereafter the tubes were incubated at 37°C until the serum controls and that tube of the antigen controls containing 1.26 units of complement were completely haemolyzed. This generally occurred within 15 to 30 minutes. Immediately afterwards the tubes were placed in the ice-box, and the first reading was made after about one hour. The second reading was made the next morning. The degree of fixation was defined by the following signs:

+ = complete fixation, no haemolysis
± = partial fixation, less than 50 per cent haemolysis
∓ = partial fixation, more than 50 per cent haemolysis
— = no fixation, complete haemolysis.

Controls

In each titration series the controls presented in Table 4 were included. The same serum was employed throughout as positive control serum. It was obtained from the person from whom the Kivinen strain of herpes virus had been isolated. The complement fixation titre of this serum was repeatedly found to be $^1/_{32}$, but in some titration series it varied one tube up or down.

THE NEUTRALIZATION TEST

With some modification, the method introduced by Burnet and Lush (33) was employed.

Strain. The Z strain was found unsuitable owing to the fact that it did not produce sufficiently large and clearly defined pock-lesions on the chorioallantoic membrane. Of the available strains, Kivinen was the best; the eleventh and twelfth egg passages were used throughout the neutralization tests.

The *dilution fluid* was sterile saline containing 20 per cent broth, the pH of which was adjusted to 7.4 with a phosphate buffer.

The *sera* were inactivated for 30 minutes at 56°C and used undiluted.

Virus suspension. Membranes infected with the Kivinen strain were diluted so as to produce 200 to 400 pocks per membrane when injected in amounts of 0.025 ml; the development of pocks was then prevented up to 90 or 100 per cent by a control serum.

The test proper. 0.2 ml of diluted virus suspension was mixed with 0.2 ml of inactivated, undiluted serum. To this mixture were added sufficient penicillin for a concentration of 500 units/ml and streptomycin 100 microgr/ml. The mixture was placed in the ice-box for 1½ to 2 hours, and thereafter volumes of 0.05 ml were inoculated onto the chorioallantoic membranes of four 12-day eggs. After incubation for 48 to 72 hours at 37°C the eggs were opened and the membranes removed. A pock count was made, and the degree to which the sera under investigation had prevented the development of pocks was estimated as compared with the effect of the herpes-positive and herpes-negative sera included as controls. Results were generally clear-cut. If the tested serum contained herpes antibody, the development of pocks was prevented to the same degree as by the positive control serum. The observation of Burnet and Lush regarding the »all or none» phenomenon was thus confirmed.

ISOLATION OF THE VIRUS

The virus was mostly isolated on the chorioallantoic membrane of 12-day eggs. Sometimes the specimen to be investigated was also inoculated intracerebrally into mice. Specimens were either collected in saline containing 20 per cent broth or in broth alone; in the latter case it was

diluted $^1/_5$ in saline before inoculation. Cerebrospinal fluids were inoculated without dilution onto the membranes. Immediately after collection, the specimens were refrigerated, and if inoculation on the same day was not possible, they were stored overnight at —10 to —15°C. Penicillin sufficient for a concentration of 500 units/ml and streptomycin 100 microgr/ml (133) were added. Thereafter volumes of 0.05 ml were inoculated onto the chorioallantoic membranes of four 12-day eggs. When mice were used, 0.03 ml was injected intracerebrally into five animals. Part of the specimen was always submitted for routine bacteriological investigation for testing the sterility. If two or more egg passages were carried out, a sample of the suspension from the first membrane passage was also submitted for bacteriological investigation.

The inoculated eggs were incubated for 3 days at 37°C. If the membranes did not then display pocks, and were normal-looking, further passages were as a rule not carried out, previous experiments having shown that typical pocks developed in abundance on the first membrane if the sample contained herpes virus. If changes were observable on the membranes, one or two additional passages were carried out. The isolated strains were identified with the use of choriollantoic neutralization tests.

In connection with the virus isolations certain observations on the preservation of various strains of herpes were made, which may be summarized here. It was found that recently isolated herpes strains deteriorated in a few days in first membrane passage material even when this was preserved in a deep-freeze. A similar observation was made by Scott, Coriell and Blank (135).

Some observations, made on three different strains, are summarized in Table 5. The Koljonen strain was isolated from herpes facialis vesicles in a patient with pneumonia. Fluid from these vesicles was mixed with

TABLE 5.

EFFECT OF STORAGE AT —10° TO —15°C ON INFECTIVITY OF 3 STRAINS OF HERPES SIMPLEX VIRUS

Strain	Passage	Storage time	Infectivity
Koljonen	Vesicle fluid	1 day	+
		3 days	+
		5 »	+
		7 »	—
	E 1	1 day	+
		3 days	+
		6 »	+
		8 »	—
	E 2	3 weeks	+
Laine	E 3	5 months	+
Armstrong	E 38	2 years	+

saline containing 20 per cent broth, buffered to a pH of 7.4. On this fluid and on materials from some of the first membrane passages, tests for infectivity were performed after storage at —10° to —15°C for the periods indicated in Table 5. The infected chorioallantoic membranes were stored, each in a test tube closed with a rubber stopper. The infectivity of the fluid from Koljonen's vesicles was titrated both on the chorioallantoic membrane and in mouse brain, whilst the infectivity of the membrane passages was titrated on the chorioallantoic membrane alone. The Laine strain was isolated from the cerebrospinal fluid in a case of encephalitis. This and the Armstrong strain were stored under the same conditions as described above. It appears from the results that under these conditions of storage, the Koljonen strain remained infective only for a short time in vesicle fluid, mixed with saline containing broth, and in the first membrane passage, but infectivity was maintained on the membrane when the virus had become well adapted to the egg. On account of these observations recently isolated strains of herpes virus were subsequently always carried through several membrane passages before being identified or stored for some length of time.

EVALUATION OF THE RELIABILITY OF THE HERPES SIMPLEX COMPLEMENT FIXATION TEST

Herpes Simplex and Mumps Complement Fixation Titres in the Same Sera

In each complement fixation series a normal egg antigen prepared from uninfected normal eggs was included as control. It is possible, however, that changes occur in the proteins of infected eggs which are not specific to the infective agent, *i.e.* in this case the herpes virus. These may possibly give non-specific fixation with some sera without being demonstrable in the controls.

In order to elucidate this point the complement fixation titres with herpes were compared with the results obtained on the same sera with another antigen from infected extra-embryonic fluid, *i.e.* mumps antigen. Titrations with an allantoic fluid mumps antigen, prepared by the technique described in 1951 by Penttinen and Halonen (114), were performed at the Virus Department of the State Serum Institute. Acute- and convalescent-phase sera, which had been submitted for serological tests for herpes or mumps, were used; these were in part unselected, in part selected inasmuch as either titre was known to have risen. The results are presented in Table 6. No correlation between herpes and mumps titres is discernible. Hence it may be concluded that unspecific antigen

TABLE 6

COMPARISON OF HERPES AND MUMPS COMPLEMENT FIXATION TITRES IN ACUTE- AND CONVALESCENT-PHASE SERA OF 31 SELECTED PATIENTS

Patient No.	Diagnosis	Serum No.	Herpes compl. fixat. titres	Mumps compl. fixat. titres
1	Stomatitis	1	<4[1]	<4
		2	8	<4
2	Stomatitis	1	<4	8
		2	<4	8
3	Poliomyelitis	1	16	<4
		2	8	<4
4	Poliomyelitis	1	<4	<4
		2	<4	<4
5	Mumps	1	<4	64
		2	<4	128
6	Encephalomeningitis	1	4	8
		2	64	8
7	Encephalomeningitis	1	<4	8
		2	<4	4
8	Encephalomeningitis	1	64	<4
		2	128	<4
9	Encephalomeningitis	1	8	8
		2	8	16
10	Encephalomeningitis	1	8	512
		2	16	256
11	Encephalomeningitis	1	<4	8
		2	<4	64
12	Encephalomeningitis	1	16	32
		2	16	128
13	Encephalomeningitis	1	<4	16
		2	<4	16
14	Encephalomeningitis	1	<4	16
		2	<4	16
15	Encephalomeningitis	1	<4	8
		2	<4	64
16	Encephalomeningitis	1	4	<4
		2	<4	<4
17	Encephalomeningitis	1	8	8
		2	8	16
		3	8	32

[1] reciprocals of serum dilutions

Patient No.	Diagnosis	Serum No.	Herpes compl. fixat. titres	Mumps compl. fixat. titres
18	Encephalomeningitis	1	32	16
		2	32	64
19	Encephalomeningitis	1	32	16
		2	32	32
20	Encephalomeningitis	1	<4	<4
		2	<4	4
21	Encephalomeningitis	1	<4	<4
		2	<4	<4
22	Encephalomeningitis	1	<4	8
		2	<4	128
23	Encephalomeningitis	1	<4	32
		2	<4	64
24	Encephalomeningitis	1	4	4
		2	8	4
		3	4	4
25	Encephalomeningitis	1	<4	<4
		2	<4	<4
26	Encephalomeningitis	1	16	128
		2	8	128
		3	8	128
27	Encephalomeningitis	1	16	16
		2	8	8
28	Encephalomeningitis	1	8	8
		2	8	4
29	Encephalomeningitis	1	32	32
		2	32	64
30	Encephalomeningitis	1	8	32
		2	16	256
31	Encephalomeningitis	1	<4	4
		2	<4	8

possibly developing in the extra-embryonic fluids of infected eggs is a factor of little significance. In any case it rarely seems to cause unspecific reactions in the complement fixation test.

Comparison of the Results Obtained by the Complement Fixation Test and the Neutralization Test

Thirty unselected sera, submitted for routine Wassermann determination, were tested for antibodies to herpes both by the

complement fixation and the neutralization technique. Neutralizing antibodies were present in 10, and these were the only ones reacting positively in the complement fixation test. The conformity of the results tallies with Hayward's observation in 1950 (77). Of her series of 95 sera, 75 reacted positively in both tests, and 19 were negative in both; one serum reacted positively in the neutralization test and negatively in the complement fixation test, but this result could not be confirmed owing to the small quantity of the sample. Dudgeon (44) used both methods in the titration of 40 sera; 30 reacted positively in both tests, although 2 sera were positive only at the dilution 1/2, which was the first in his titration series. Hayward used dilutions from 1/8 and regarded as negative in the complement fixation test all the sera which gave no fixation at this dilution. In the present investigation one serum alone was positive only at the first dilution, 1/4. In the series of Holzel *et al.* (79), including 52 sera reacting positively in the neutralization test, 2 were negative in the complement fixation test; 23 sera reacted negatively in the neutralization test, and these were negative also in the complement fixation test.

Gajdusek, Robbins and Robbins (61) compared complement fixation titres with the results obtained in the neutralization test on mice. As was pointed out on page 20, these investigators held that the complement fixation test always yields positive results when the neutralization index is high, and negative when the latter is very low. In the present investigation no attempts were made to estimate the ratio between the titres of neutralizing and complement-fixing antibodies, since the chorioallantoic neutralization test is not a good aid where accurate determinations of herpetic antibody titres are involved.

Results of Herpes Complement Fixation Tests on Sera Distributed According to the Herpes History

Of 126 medical students from whom serum samples were collected, 21 stated that they sometimes had or occasionally had had herpetic vesicles on the lips or around the mouth. The complement fixation results obtained on these sera are presented in Table 7. All the 21 students with a positive history had complement-fixing herpes antibodies, but antibodies were also present in 31 of the 105

TABLE 7

HERPES COMPLEMENT FIXATION TITRES IN THE SERA OF 126 MEDICAL STUDENTS WITH POSITIVE OR NEGATIVE HERPES HISTORY

Herpes history	Complement fixation test						
	Negative at serum. dil. 1/4	Positive at final serum dil. of					
		1/4	1/8	1/16	1/32	1/64	1/128
+ 21	0	2	2	4	8	2	3
—105	74	3	9	7	10	1	1

students with a negative history of herpes; *i.e.* of the 52 subjects who had complement-fixing antibodies, 31, or 60 per cent, were not conscious of ever having had herpetic vesicles. This finding is at variance with the results obtained in 1932 by Brain and in 1949 by Hayward. Using the complement fixation test with antigen prepared from guinea-pig pads, Brain (27) investigated 10 subjects, whose herpes histories were known, for herpes antibodies. He reported that out of these, 4 with a positive history had complement-fixing antibodies, whilst the remaining 6, who had a negative history, were negative in the complement fixation test. Hayward (76), too, stated that only the sera from individuals with a positive history of herpes infection gave positive results in the complement fixation test.

In the present investigation the average complement fixation titre in the group with a positive history of herpes was higher than in the group with a negative history of herpes. On account of the relatively small number of sera investigated and the wide dispersion of the titres it is difficult to draw any conclusions, however, and the same holds with regard to the fact that in the cases where recurrent herpetic vesicles were reported, the average titre was somewhat higher than in those where there was a history of vesicles which had not recurred for some years.

Variations in the Complement-Fixing Antibody Titres in Diseases not due to the Virus of Herpes Simplex

In order to substantiate the usefulness of the complement fixation test in the serological diagnosis of herpetic diseases, it seemed important to investigate the possible variations in the titres of complement-fixing herpes antibody in clinical conditions

TABLE 8

HERPES COMPLEMENT FIXATION TITRES IN ACUTE- AND CONVALESCENT-PHASE SERA OF 45 PATIENTS WITH PNEUMONIA, POLIOMYELITIS AND MUMPS

Patient No.	Diagnosis	Period after onset	Herpes complement fixation titres
1	Pneumonia	2 days	16[1]
		23 days	16
2	Pneumonia	4 days	16
		12 days	32
3	Pneumonia	3 days	8
		2 months	8
4	Pneumonia	3 days	<4
		25 days	<4
5	Pneumonia	5 days	<4
		2 months	<4
6	Pneumonia	2 days	64
		17 days	64
7	Pneumonia	4 days	32
		25 days	32
8	Pneumonia	3 days	32
		14 days	16
9	Pneumonia	2 days	16
		10 days	32
10	Pneumonia	5 days	32
		15 days	32
11	Pneumonia	10 days	16
		24 days	16
12	Pneumonia	2 days	<4
		14 days	<4
13	Pneumonia	5 days	16
		16 days	16
14	Pneumonia	2 days	32
		10 days	32
15	Poliomyelitis	5 days	8
		5 weeks	8
16	Poliomyelitis	22 days	<4
		2 months	<4
17	Poliomyelitis	9 days	<4
		24 days	<4
18	Poliomyelitis	4 days	<4
		17 days	<4
19	Poliomyelitis	21 days	<4
		31 days	<4
20	Poliomyelitis	4 days	16
		18 days	16
21	Poliomyelitis	11 days	4
		20 days	4
22	Poliomyelitis	5 days	<4
		16 days	<4

[1] reciprocals of serum dilutions

(Cont.)

Patient No.	Diagnosis	Period after onset	Herpes complement fixation titres
23	Poliomyelitis	3 days	<4
		14 days	<4
24	Poliomyelitis	6 days	32
		20 days	32
		24 days	64
25	Poliomyelitis	4 days	16
		17 days	16
		20 days	16
26	Poliomyelitis	2 days	32
		9 days	32
27	Poliomyelitis	5 days	16
		13 days	16
28	Poliomyelitis	10 days	<4
		23 days	<4
29	Poliomyelitis	8 days	32
		21 days	32
30	Poliomyelitis	5 days	16
		16 days	8
31	Poliomyelitis	4 days	32
		14 days	16
32	Poliomyelitis	8 days	<4
		17 days	<4
33	Poliomyelitis	20 days	<4
		29 days	<4
34	Poliomyelitis	5 days	<4
		15 days	<4
35	Poliomyelitis	3 days	<4
		13 days	<4
36	Poliomyelitis	5 days	<4
		17 days	<4
37	Poliomyelitis	4 days	<4
		19 days	<4
38	Mumps	6 days	8
		16 days	8
39	Mumps	7 days	<4
		23 days	<4
40	Mumps	5 days	<4
		15 days	<4
41	Mumps	5 days	<4
		18 days	<4
42	Mumps	10 days	4
		32 days	4
43	Mumps	5 days	4
		13 days	4
44	Mumps	9 days	<4
		21 days	<4
45	Mumps	4 days	<4
		14 days	<4

not due to herpes virus, especially where carriers of this agent are apt to develop herpetic vesicles. If, in such patients, the herpes titres were found to rise significantly, it would be difficult to distinguish serologically between conditions due to a primary herpetic infection and illness accompanied by the recurrence of herpes simplex.

Complement fixation tests for herpes were therefore performed on the acute- and convalescent-phase sera of 14 patients with pneumonia, 23 with poliomyelitis and 8 with mumps. The results are shown in Table 8. During the course of the illness the herpes titres remained constant in 39 out of these 45 cases; in 3 cases (nos. 2, 9, 24) they rose, and in 3 (nos. 8, 30, 31) they dropped. In no case was the increase in titre more than twofold. Hence it appears that in non-herpetic diseases only minor changes are demonstrable in the complement-fixing herpes antibody titres. The same observation was made on the person from whose lip the Kivinen strain of herpes simplex had been isolated (cf. page 25). Over a period of nearly two years, serum specimens were recovered from her immediately after the development of vesicles, a couple of weeks, and some months later. All these sera showed the same titre of complement-fixing herpes antibody.

DISCUSSION

One of the problems involved in the study of the herpes complement fixation test is the question of whether more than one strain should be used in the preparation of antigen. The point at issue is whether serological differences between some strains are so great that it is not possible to demonstrate the presence of antibody to all strains with antigen prepared using one strain only. In general the strains isolated in different herpes simplex infections have been regarded as serologically similar. Recently Womack and Hunt (cf. page 20), however, have stated that striking serological differences sometimes exist between strains of herpes simplex. Previously Hayward (76), using a similar technique, investigated the antigenic properties of four recently isolated strains of herpes virus. There were considerable differences in complement fixation titres between the sera of experimental animals immunized with

these strains; differences were not demonstrable, however, using the sera of the subjects from whom the strains in question had been isolated. Hayward stated that these tests »give no indication of strain specificity among the four viruses», and she concluded that no antigenic differences between these strains could be demonstrated with the method employed.

In the present investigation it was found that all subjects with a history of herpetic vesicles had complement-fixing antibodies against antigen prepared with one strain of herpes virus. This seems to indicate that the antigenic differences between various strains cannot be very great. It is possible, however, that the Z strain employed, like the O'C strain of Womack and Hunt, is a particularly efficient producer of antigen, and that antibodies to antigenically slightly different strains are therefore demonstrable with Z strain antigen.

However, certain differences between various strains of herpes simplex virus were observed in the present investigation also, the most striking being a varying capacity to produce complement-fixing herpetic antigen in embryonated eggs, in particular in the extra-embryonic fluids. Hence the choice of a suitable strain of herpes virus for the preparation of antigen seems to be essential.

Furthermore it was found that the success of the herpes complement fixation test is dependent on the time of fixation between antigen and antibody. This observation, made with the use of extra-embryonic fluid antigens, tallies with Bedson and Bland's (cf. page 17) finding with antigen prepared with herpes-infected guinea-pig pads. When the fixation time was short (one hour at 37°C), negative results were obtained in the present tests, or the titres were very low, whilst titres up to 1/128 were simultaneously obtained on the same sera using prolonged fixation (20 hours at 4°C).

These two findings, *i.e.* the varying capacity of different strains of herpes to produce complement-fixing antigen and the importance of prolonged fixation, seem to explain most of the negative results previously reported in herpes complement fixation tests. Gajdusek, Robbins and Robbins (61) suggested that the unsatisfactory results described by Afzelius-Alm were due to his having used a strain of herpes virus which gave a poor complement fixingantigen.

In view of the present results, the fact that he allowed fixation between antigen and serum for only 45 minutes at 37°C would also seem to be responsible for his low complement fixation titres.

The reliability of the complement fixation test for herpes was investigated with the use of amniotic-allantoic fluid antigen prepared with the Z strain. It was found that the herpes titres bore no relation to the complement fixation titres obtained on the same sera employing extra-embryonic fluid antigen obtained with mumps virus. Furthermore it was found that the results of the herpes complement fixation test tallied with those obtained with the neutralization test, and that, of the subjects investigated, all with a history of herpetic vesicles had antibodies to herpes. When the acute- and convalescent-phase sera of patients with pneumonia, poliomyelitis and mumps were tested for the presence of herpes antibodies by the complement fixation technique, there was never a rise in titre by more than twofold. Hence it may be concluded that a fourfold rise in herpes antibody titre would provide a reasonable ground for referring an illness to herpetic infection.

The conformity of the results obtained in neutralization and complement fixation tests, reported by previous workers too (44, 61, 77, 79), is at variance with the finding in the majority of similar investigations on other viruses. This is, perhaps, consequent on the peculiar infective mechanism of herpes simplex. During the acute stage of virus diseases there is a more or less simultaneous increase in both neutralizing and complement-fixing antibodies. The latter begin to disappear from the serum sooner than the neutralizing antibodies, and the two serological methods therefore yield different results at a later stage. In diseases due to the herpes virus, on the other hand, those who have suffered from a primary infection become carriers of the virus and develop recurrent herpetic vesicles. Owing to repeated stimulation neither kind of herpes antibody decreases, not even the complement-fixing antibodies. Hence it seems that, when the purpose is to estimate what proportion of a population have been infected with a certain virus, the complement fixation test is a more suitable aid with regard to herpes simplex than with regard to other viruses.

II. STUDIES ON HERPES SIMPLEX ANTIBODIES.

MATERIAL

In 85 series of herpes simplex complement fixation tests, 2292 titrations were carried out on 1375 specimens of serum from 1050 subjects. The present study is based on the results obtained on 1040 of these specimens, recovered from 842 subjects.

The following sera representing various age groups were used: cord sera obtained from the Women's Clinic of the University of Helsinki; children's sera from the Children's Clinic of the University of Helsinki and from the Aurora Hospital (the municipal hospital for infectious diseases in Helsinki) submitted for routine Wassermann determination; sera obtained from the Sofianlehto residential nursery; sera of conscripts from the garrison of Helsinki [1]; sera of medical students at the University of Helsinki, and sera from inmates of the Old People's Home in Helsinki.

The sera representing various diseases were obtained from patients at the Aurora Hospital, the Children's Clinic and the Dermatological Clinic of the University of Helsinki, the Maria Hospital, the 1st Central Military Hospital, the Children's Clinic of the University of Turku, the County Hospital at Oulu, the Central Hospital of Central Finland, and the hospitals for infectious diseases at Turku, Tampere and Pori. These specimens had been submitted to the Department of Virology of the Universityof Helsinki and the Virus Department of the State Serum Institute for serological investigations.

DISPERSION OF ANTIBODY TITRE VALUES

Fig. 1 shows the percentile range of the complement fixation titres of all the herpes-positive sera investigated. The »all or none» phenomenon revealed by the chorioallantoic neutralization test (cf. page 31) is not observable in these results, which display a wide dispersion, though the groups 1/16 and 1/32 constitute over 50 per cent of all positive sera. The highest complement fixation titre in this investigation, *viz.* 1/512, was obtained on the serum of a child admitted with a diagnosis of »infectio acuta».

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[1] These sera were kindly placed at the writer's disposal by Dr. Pirkko Pohjanpelto.

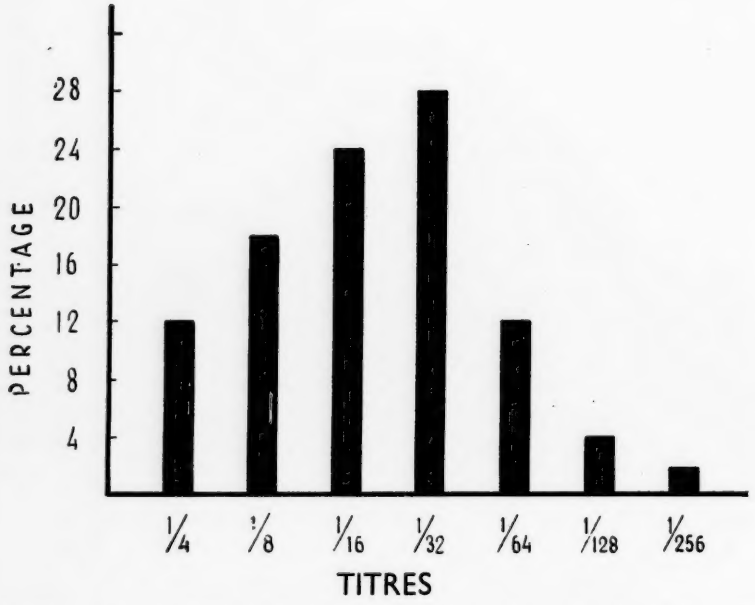

Fig. 1. Dispersion of herpes complement fixation titres in all tested herpes-positive sera.

AGE INCIDENCE OF ANTIBODIES

Previous Investigations

The first to compare the incidence of herpes antibodies in children and adults were Zinsser and Tang, in 1929 (166). Using the neutralization test on rabbit brain they found that 11 normal adults out of 17, or 59 per cent, and 10, or 43 per cent, out of 23 children admitted with various complaints to a children's hospital, had antibodies to herpes. Of the children, 16 were under 7 years of age; of these, 25 per cent had antibodies. Weyer (158) studied the incidence of herpes antibodies in various age groups with the aid of the neutralization test on mice. In his series antibody was present in 14 per cent of those aged 0 to 5 years, and in 38 per cent in the group 5 to 10 years. The incidence gradually rose with age, being 90 per cent in the groups 15 to 20, and 20 to 25 years. Thereafter it decreased, the percentage figures being 65 in subjects aged 40 to 45, and 54 in those over 45 years old. Each age group was represented by 10 to 28 sera. During eleven months Anderson and

Hamilton (8), using the chorioallantoic neutralization test, observed the development of herpes antibodies in 51 children under 3 years old in a Melbourne orphanage, who were not known to have had stomatitis or herpetic vesicles. During the period of observation, antibodies to herpes developed in 29. In 7 children the neutralization titres remained high throughout this time. Anderson and Hamilton assumed that these children had already suffered from a subclinical infection with herpes virus when the first sample was drawn. In 10 children, whose first serum specimen displayed a high antibody level, subsequent tests gave lower titres. In these cases the antibodies had obviously been transferred from the mother and disappeared completely when the children reached the age of 3 to 15 months. Przesmycki, Prazmowski and Semkov (119) also used the chorioallantoic neutralization test when investigating 100 sera, representing different age groups, for the presence of herpes antibody. In their material only some of the sera of those aged 0 to 1 year contained antibody. In the older groups the number of herpes-positive sera increased, the highest incidence being noted in the group 11 to 15 years, after which it was again somewhat lower. In 1953 Buddingh *et al.* (31), using the chorioallantoic neutralization technique, investigated the age incidence of herpes antibody in specimens submitted in Louisiana for routine Wassermann and Kahn tests. In their material, 6-month-old and younger white children had antibodies in 79 per cent of cases, the corresponding percentage figure for Negroes being 93. The high incidence was referred to a transfer of antibodies from the mothers, and the writers state that in cord sera the antibody titres were largely the same as in the mothers. In the age group 7 months to 2 years 43 per cent of the white children and 71 per cent of the Negro children had antibodies; in the group 3 to 14 years the corresponding figures were 85 and 86 per cent, and in the group 15 and more years they were 90 and 96 per cent. The number of sera in the different groups varied from 27 to 171. In Manchester, Holzel *et al.* (79), using the complement fixation test with chorioallantoic antigen, tested the sera of 325 subjects of various ages for the presence of herpes antibody. Of a total of 32 cord sera, 78 per cent were positive; in the group 0 to 1 month the incidence was about the same, or 77 per cent. Then there was a gradual decline, the percentage figures being 25 for the group 1 to 2 months and 13 for the group

6 to 12 months. Thereafter the incidence rose again, being 30 per cent in those aged 1 to 2 years, 63 per cent in the group 5 to 15, and 86 per cent in those over 15 years old; the mean age of the last-mentioned group was 36 years.

The presence of herpes antibodies in adults was determined by Andrewes and Carmichael in 1930 (10) with the use of the neutralization test on rabbit skin; a positive result was obtained in 75 per cent of cases. In 1936 Hudson, Cook and Adair (81), using mice, demonstrated the presence of neutralizing herpes antibodies in 87 per cent of the women and 82 per cent of the men in a series of adults. In 1932, when testing a series of 52 unselected adult sera for complement-fixing herpes antibodies with a guinea-pig pad antigen, Brain (27) was able to demonstrate fixation in 45 per cent. Adult sera were also investigated by Hayward (77), who found that 80 per cent were herpes-positive.

Own Investigations

The incidence of herpes antibodies in different age groups was investigated on 599 sera, using the complement fixation test. The results are demonstrated in Table 9, where the sera showing unspecific fixation with the control antigen are also listed. Of 28 cord sera 7 had this property; of the remaining 21, 12 were herpes-positive and 9 were negative. Antibody was thus present in 57 per cent. In this group the dispersion of the antibody titres was not so wide as in the other groups, the titre being 1/64 in almost all the positive sera. The incidence of antibody was about the same in the group under 3 months old (56 per cent). The titres were very low, 1/4 and 1/8, in particular in children aged 2 to 3 months. Of 81 sera investigated in the group 3 to 12 months, only 2 were positive; in both cases the child was a little under 4 months old. The titres were 1/4 and 1/32, respectively. After the first year the relative number of herpes-positive sera rose, the percentage figures being 25 in the age group 1 to 3 years, 38 in the group 3 to 7 years, and 46 in the group 7 to 15 years. Of 163 conscripts with a mean age of 20 years, 73, or 45 per cent, had antibodies. The incidence was 41 per cent amongst medical students, and 40 per cent amongst old people with a mean age of 77 years.

The incidence of antibody in the cord sera and in the group

TABLE 9

COMPLEMENT-FIXING HERPES ANTIBODIES IN DIFFERENT AGE GROUPS

Age group	Total number of sera tested	Number of positive sera	Number of negative sera	% positive	Number of sera giving non-specific fixat. with control antigen
Cord sera	28	12	9	57	7
0—3 months	33	18	14	56	1
3—12 months	81	2	79	2	0
1—3 years	59	15	44	25	0
3—7 years	37	14	23	38	0
7—15 years	42	19	22	46	1
Conscripts (mean age 20 years)	163	73	90	45	0
Medical students (mean age 24 years)	126	52	74	41	0
Old people (mean age 77 years)	30	12	18	40	0

0 to 3 months (57 and 56 per cent, respectively) seems high in comparison with the incidence in adults, which was 40 to 45 per cent. It should be emphasized, however, that the middle-aged are entirely unrepresented in this material. The difference may also be due to the fact that all social groups are not, perhaps, represented in equal numbers in the different age groups. As will be explained later, this circumstance may have influenced the results to a considerable degree.

In all age groups the incidence is lower than what has generally been established elsewhere. It seems that the incidence of herpes-positive sera varies in different countries. In Afzelius-Alm's (4) material from Sweden, 79 per cent of sera contained neutralizing herpes antibodies, whilst an investigation by the same technique of a series from the U.S. Army personnel revealed an incidence of 53 per cent. Even in the same country the difference between social groups may be great. In Australia, Burnet (35) found that 37 per cent of university graduates had herpes antibodies, whilst the incidence was 59 per cent amongst non-graduate laboratory workers and 93 per cent amongst public hospital patients. The different

results obtained by Afzelius-Alm in his Swedish and American series may, perhaps, also be referred to social factors. In England, differences in the incidence of antibody between social groups were demonstrated by Andrewes and Carmichael (10).

In the present investigation, it cannot be claimed that the material in the different age groups is representative of the whole Finnish population of the corresponding ages, but with regard to the group consisting of sera submitted for serological tests for stomatitis, eczema herpeticum, encephalomeningitis and other diseases possibly caused by the virus of herpes simplex, it gives a fairly good idea of the development of herpes antibody. Furthermore it should be borne in mind that over fifty per cent of the sera in the age group 0 to 15 years were obtained from the Children's Clinic of the University of Helsinki, the patients of which are almost evenly distributed among the different social groups.

ANTIBODIES IN PATIENTS WITH STOMATITIS

Previous Investigations

As soon as the role of herpes virus in the pathogenesis of herpetic vesicles developing on the mucous membranes had been disclosed, attention was directed to the possibility that inflammations of the mucous membrane of the mouth were also caused by this agent. In 1932 and 1933 (99, 164) virus of herpes simplex from adult patients with stomatitis was isolated on the cornea of the rabbit, but considering that by the same technique it had also been isolated from the mouths of normal subjects, the virus of herpes simplex could not be credited with etiological significance in stomatitis purely on the ground of these few observations.

Investigating extensive series, Dodd, Johnston and Buddingh (41), and Gottron (69), in 1938 reported the isolation of herpes virus in many cases of stomatitis in children; the isolated strains had also been identified both histologically and immunologically. On account of the clinical picture they regarded herpetic stomatitis as a clearly defined disease, caused by the virus of herpes simplex. Conclusive evidence that stomatitis was due to herpes virus was finally produced in 1939 by Burnet and Lush (34) and Burnet and Williams (35). They demonstrated the development of neutralizing herpes antibody in the sera of children suffering from stomatitis,

and succeeded simultaneously in isolating herpes virus from the mouths of the patients.

In 1941 Scott, Steigman and Convey (136) published a report on 33 cases of acute infectious gingivostomatitis in children. In 15 of these they had isolated herpes virus, and in 21 the development of neutralizing herpes antibody had been demonstrated. The age of the children varied from 12 months to 12 years; 40 per cent were under 3 years, and 70 per cent under 6 years old. This material included three familial epidemics with many children from the same family simultaneously suffering from herpetic stomatitis. From 15 out of 23 patients with acute infectious gingivostomatitis Black (22) was able to isolate herpes virus; furthermore he demonstrated the development of herpes antibody in many of these cases. The age of the patients varied from 1 to 15 years, most of them being 1½ to 2 years old. Over a period of eleven months Anderson and Hamilton (8) followed the occurrence of stomatitis and the development of herpes antibody in 51 children under 3 years old in a Melbourne orphanage; specimens for serological investigation were four times recovered during this period. Twenty of these children suffered from stomatitis during the time of observation. In all the sera collected prior to the onset of disease, neutralizing herpes antibody was lacking, whilst the specimens obtained after recovery were invariably positive. Rogers *et al.* (122), in 1949, reported on 3 cases of gingivostomatitis in adults, where the herpes antibody level rose. Dudgeon (44) noted an increase in herpes complement-fixing antibody titres in 6 children suffering from stomatitis. Investigating 9 cases of acute gingivostomatitis, Gajdusek, Robbins and Robbins (61) also demonstrated an increase in complement-fixing herpes antibodies; their series included one adult, the remainder being children under 5. In 1953, Buddingh *et al.* (31) followed the development of neutralizing antibodies in serum specimens from 12 patients with herpetic stomatitis. These workers found that determinable amounts were present in the serum from the fourth to seventh day after onset of the disease, and that the maximum titre was reached by the third to fourth week. However, the level and the time of development of antibody varied considerably in the different patients. An average of 23 days after onset, the virus of herpes simplex could still be isolated from the mouths of the patients, the extremes being 4 and 42 days.

Own Investigations

The present series consists of 28 patients with stomatitis, from whom one or several specimens of serum were obtained at different stages of the illness. The clinical picture, in particular the severity of the disease, varied in these cases, but features common to all were sudden fever and aphthous or vesicular inflammation of the mucous membrane of the mouth. As a rule recovery occurred within one or two weeks. The age of the patients varied between 1½ and 36 years.

The herpes complement fixation titres of the sera are presented in Table 10. It is difficult to summarize the results owing to the fact that samples were collected from the patients at very different phases of the illness, and that individual variations in the time of development of herpes antibody are considerable. Two or more specimens were obtained in 18 cases, and only in 12 of these (nos. 2, 8, 9, 14, 15, 17, 18, 19, 24, 26, 27, 28) was the first sample recovered during the acute stage, whilst the second was obtained at least two weeks after the onset of the disease. The sera of 3 out of these 12 patients showed a fourfold or greater increase in complement-fixing herpes antibody (nos. 2, 8, 24). In all three cases the first specimen was negative, and titres for the second varied from 1/8 to 1/32. The clinical picture and the laboratory findings of patient no. 2 are presented in case report no. 1. This case has previously been reported on by Halonen and Penttinen (75). Furthermore the first sample from patient no. 22, collected three days after onset, gave a negative response; in the second sample, obtained five days later, the titre was 1/4. In patient no. 23 the titres were 1/16 in a specimen recovered eight days after onset and 1/32 four days later. From patient no. 16 a sample obtained one and a half months after the onset of illness showed a titre of 1/32; two weeks, and one and a half months later the titre was 1/16. Out of the 14 patients (nos. 2, 3, 8, 9, 14, 15, 16, 17, 18, 19, 24, 26, 27, 28) from whom samples were obtained a minimum of two weeks after onset, 8 had no herpes antibody at this time.

A definite increase in herpes antibody was thus demonstrated only in one-quarter of the cases in which it might have been expected, and even if the patients displaying a twofold rise in titre are included, the group with increased titres constitutes no more

TABLE 10

RESULTS OF HERPES COMPLEMENT FIXATION TESTS IN PATIENTS WITH STOMATITIS

Patient No.	Age	Period after onset	Herpes complement fixation titres
1	12 years	11 days	<4 [1]
2	6 years	5 days	<4
		18 days	32
3	16 years	1 month	32
4	3 years	8 days	<4
5	10 months	10 days	<4
6	4 years	7 days	<4
7	7 years	4 days	<4
		7 days	<4
8	5 years	5 days	<4
		18 days	16
9	4 years	5 days	8
		15 days	8
10	1.5 years	6 days	<4
		10 days	<4
11	23 years	6 days	<4
12	3 years	6 days	<4
13	1 year	4 days	<4
14	25 years	4 days	<4
		17 days	<4
15	7 years	9 days	<4
		17 days	<4
		1 month	<4
16	9 years	1.5 months	32
		2 months	16
		3 months	16
17	36 years	4 days	<4
		18 days	<4
18	9 months	3 days	<4
		23 days	<4
19	2 years	7 days	<4
		16 days	<4
20	2 years	13 days	32
21	3 years	4 days	<4
		9 days	<4
22	2 years	3 days	<4
		8 days	4
23	5 years	8 days	16
		12 days	32

[1] reciprocals of serum dilutions

(Cont.)

Patient No.	Age	Period after onset	Herpes complement fixation titres
24	8 years	7 days	<4
		17 days	8
25	3 years	2 days	<4
26	1.5 years	3 days	<4
		14 days	<4
27	7 years	3 days	<4
		20 days	<4
28	6 years	4 days	<4
		14 days	<4
		19 days	<4

than one-third of the series. On the other hand, of the patients from whom specimens were obtained so late that there would obviously have been sufficient time for complement-fixing herpes antibody to develop, more than half had negative sera.

ANTIBODIES IN PATIENTS WITH ACUTE ENCEPHALOMENINGITIS

Previous Investigations

As stated on pages 12 and 13, many cases of encephalitis and aseptic meningitis have been described in which either the virus of herpes simplex has been isolated, or intranuclear inclusions typical of herpes have been demonstrated in the brain tissue of the patient; in some cases both findings have been made. Of acute diseases of the central nervous system caused by herpes virus, only a few cases are on record (3, 4, 138) in which an increase in herpes antibody titres has been demonstrated, and fatal herpetic encephalitis showing a rise in antibody titre has been reported only once (115). The absence of further evidence with regard to the latter condition is not surprising, considering that the downhill course in these cases is generally too sudden for the development of antibody.

In 1951 Afzelius-Alm (4) reported an investigation of acute- and convalescent-phase sera from patients with acute aseptic encephalomeningitis, which he had also tested for herpes antibody, employing either the chorioallantoic neutralization, or the complement fixation test. The material was collected in Gothen-

burg and from U.S. Army personnel. In 3 out of 103 cases investigated by the complement fixation method, and in 7 out of 145 examined by the neutralization test, the antibody level had risen. In one case, tested by both techniques, complement-fixing and neutralizing antibodies had both developed. Afzelius-Alm concluded that some 7 per cent of all cases of non-epidemic, acute, aseptic encephalomeningitis may be referred to a primary herpetic infection. In his series the age of the patients varied from 15 to 47 years, the majority being between 20 and 30.

In 1952 Scott *et al.* (138) investigated herpetic infections in children, paying special attention to unusual clinical manifestations. Their series included a 4-year-old boy with encephalitis, who had been admitted on suspicion of brain tumour or brain abscess; in this case they demonstrated a rise in both neutralizing and complement-fixing herpes antibody titres during the course of the illness.

In 1953 a report on aseptic meningitis was published by Adair, Gauld and Smadel (3). They had tested a large series of sporadic cases of acute aseptic meningitis for antibodies to the agents of lymphocytic choriomeningitis, mumps, herpes simplex and leptospirosis. They regarded the virus of herpes simplex as the causative factor in about 5 per cent of the selected cases of acute aseptic meningitis which previously had been studied serologically and found negative for LCM and mumps infections. In the whole series, 12 per cent of cases were caused by the mumps virus and 9 per cent by the LCM virus. In 4 of their 8 cases of herpetic meningitis there was a rise in both complement-fixing and neutralizing antibody; in one the neutralizing antibody titre persisted on the same level, whilst complement-fixing antibodies increased; in 2, neutralizing antibodies increased whilst complement fixation titres remained the same, and in one case the titre of neutralizing antibody rose whilst specimens drawn 12 and 23 days after the onset of illness gave a negative response in the complement fixation test.

Penttinen *et al.* (115) demonstrated an increase in complement-fixing herpes antibody in a child who developed symptoms of encephalomeningitis at the age of two weeks. This case was interesting inasmuch as the encephalitis became chronic, when the patient had recovered from the acute phase; after four months the child died of aspiration pneumonia resulting from an exacerbation of the general condition. The diagnosis of herpetic encephalomen-

ingitis was confirmed by isolation of the virus of herpes simplex from dermal vesicles and from the cerebrospinal fluid, and by the demonstration of intranuclear inclusions in the brain tissue.

Own Investigations

In the present study, acute encephalomeningitis has been used as a general name for cases admitted to various hospitals with a diagnosis of meningitis serosa (lymphocytica, virosa, aseptica), meningoencephalitis acuta or encephalitis acuta. This has been necessary owing to the fact that the criteria employed in distinguishing these conditions are not the same in all hospitals, and case reports do not always contain sufficient neurological data for sub-division into adequate groups. The present material includes 91 sporadic cases of acute encephalomeningitis, in which two or more serum specimens were collected during the years 1949–1954. Over half of all samples were obtained from the Aurora Hospital.

The age of the patients varied between 15 days and 64 years. The age distribution was 20 in the group 0 to 5 years, 37 in the group 5 to 15 years, 13 in the group 15 to 25, and 21 over 25 years with a mean age of 38 years. The clinical picture varied from slight symptoms of aseptic meningitis to the violent manifestations of fatal encephalomeningitis.

Table 11 shows the herpes complement fixation titres obtained on the sera in these 91 cases. Different specimens from the same patient were titrated as one series, and if this showed an increase in antibodies, the results were checked by another titration series. In 6 cases (nos. 4, 10, 43, 58, 67, 68) the rise in titre was fourfold or greater. In 4 of these (nos. 4, 10, 43, 68) the titre was 1/4 or 1/<4 in the first specimen, and in case no. 58 it was 1/8, but in the latter the first sample was not obtained until 15 days after the onset of illness. Patient no. 67 alone had definite herpes titres at the initial stage of the disease, the titre being 1/16 in a sample collected five days after onset. A twofold rise in titre was noted in 6 patients (nos. 14, 24, 30, 31, 49, 81), all of whom had herpes antibodies in the acute phase.

In 13 cases the second specimen was recovered as early as the second week of illness, or both specimens were collected so late

TABLE 11

RESULTS OF HERPES COMPLEMENT FIXATION TESTS IN PATIENTS WITH ACUTE ENCEPHALOMENINGITIS

Patient No.	Age	Period after onset	Complement fixation titres
1	33 years	10 days	<4 [1]
		31 days	<4
2	21 years	2 days	4
		14 days	4
		29 days	4
3	10 years	2 days	4
		16 days	4
4	4 years	4 days	<4
		18 days	8
5	26 years	6 days	<4
		8 days	<4
		22 days	<4
6	5 years	7 days	8
		21 days	8
7	14 days	2 days	<4
		12 days	<4
		1.5 months	<4
8	19 years	1 day	<4
		14 days	<4
9	56 years	14 days	128
		28 days	128
10	38 years	4 days	4
		2 months	128
11	18 years	3 days	<4
		15 days	<4
		1.5 months	<4
12	6 months	16 days	<4
		26 days	<4
13	12 years	2 days	<4
		14 days	<4
		1.5 months	<4
14	28 years	2 days	8
		10 days	16
15	15 years	17 days	<4
		1.5 months	<4
16	7 years	3 days	8
		14 days	8
17	32 years	14 days	32
		35 days	32
18	9 years	2 months	<4
		3 months	<4
19	3 months	4 days	<4
		14 days	<4

[1] reciprocals of serum dilutions

(Cont.)

Patient No.	Age	Period after onset	Complement fixation titres
20	7 years	4 days	<4
		22 days	<4
21	9 years	2 days	<4
		10 days	<4
22	10 years	2 days	<4
		14 days	<4
23	14 years	4 days	<4
		19 days	<4
24	9 years	4 days	4
		21 days	8
25	7 years	7 days	<4
		24 days	<4
26	12 years	12 days	<4
		17 days	<4
		39 days	<4
27	49 years	8 days	4
		20 days	8
		31 days	4
28	5 years	2 days	<4
		21 days	<4
29	14 years	5 days	<4
		2 months	<4
30	11 years	4 days	64
		2 months	128
31	17 years	9 days	16
		20 days	32
32	12 years	4 days	<4
		19 days	<4
33	27 years	9 days	<4
		17 days	<4
		32 days	<4
34	36 years	5 days	<4
		16 days	<4
		32 days	<4
35	5 years	6 days	<4
		21 days	<4
		30 days	<4
36	38 years	16 days	8
		27 days	8
		1.5 months	8
37	13 years	5 days	32
		14 days	32
38	9 years	1 day	<4
		8 days	<4
39	18 years	4 days	4
		12 days	4
		25 days	4

(Cont.)

Patient No.	Age	Period after onset	Complement fixation titres
40	35 years	13 days	32
		32 days	32
41	3 years	6 days	<4
		17 days	<4
42	15 years	4 days	<4
		16 days	<4
43	7 years	3 days	<4
		14 days	16
44	1 year	1 day	<4
		12 days	<4
		27 days	<4
45	7 year	21 days	<4
		1.5 months	<4
46	7 years	5 days	8
		9 days	8
47	13 years	4 days	<4
		11 days	<4
48	20 years	5 days	8
		15 days	8
49	26 years	1 day	8
		8 days	16
50	13 years	1 day	<4
		12 days	<4
51	7 years	2 days	<4
		9 days	<4
52	45 years	5 days	<4
		14 days	<4
53	3 years	3 days	<4
		15 days	<4
54	46 years	4 days	32
		15 days	32
55	20 years	3 days	<4
		24 days	<4
56	8 years	4 days	<4
		12 days	<4
57	13 years	2 days	<4
		14 days	<4
58	15 days	14 days	8
		24 days	16
		2 months	64
59	5 years	3 days	64
		14 days	64
60	9 years	5 days	<4
		14 days	<4
61	8 months	4 days	<4
		15 days	<4

(Cont.)

Patient No.	Age	Period after onset	Complement fixation titres
62	25 years	19 days	8
		2 months	8
63	27 years	1 months	<4
		2 months	<4
64	1.5 years	6 days	<4
		16 days	<4
65	11 years	1.5 months	16
		2 months	16
66	2 years	3 months	<4
		4 months	<4
67	11 years	7 days	16
		1 month	32
		2 months	64
68	64 years	9 days	<4
		27 days	16
69	24 years	24 days	<4
		1.5 months	<4
70	58 years	11 days	64
		1.5 months	32
		2 months	64
71	4 years	14 days	8
		22 days	8
72	8 years	1 day	<4
		8 days	<4
73	18 years	2 days	<4
		20 days	<4
74	20 years	11 days	<4
		18 days	<4
75	5 years	2 days	<4
		10 days	<4
76	5 years	2 days	<4
		14 days	<4
77	6 years	3 days	<4
		14 days	<4
78	40 years	7 days	32
		21 days	32
		38 days	32
79	10 years	9 days	<4
		19 days	<4
80	8 years	4 days	<4
		20 days	<4
81	4 years	6 days	16
		16 days	32
82	34 years	4 days	16
		14 days	16
		2.5 months	16

(Cont.)

Patient No.	Age	Period after onset	Complement fixation titres
83	26 years	3 days	<4
		18 days	<4
84	4.5 years	3 days	<4
		20 days	<4
85	17 years	4 days	<4
		20 days	<4
86	18 years	5 days	<4
		27 days	<4
87	31 years	4 days	<4
		24 days	<4
88	5 years	6 days	<4
		16 days	<4
89	5 years	3 days	8
		14 days	8
90	7 years	9 days	<4
		20 days	<4
91	6 years	4 days	<4
		14 days	<4
		18 days	<4

that a possible previous increase in antibody could no longer be demonstrated. When these are eliminated, a series of 78 comparable cases remains, in which the 6 cases showing a definite increase in antibody constitute about 8 per cent.

The disease was fatal in cases nos. 58 and 68. Patient no. 58 has been reported on by Penttinen *et al.* (115); the diagnosis was in this case confirmed by isolation of the virus and by demonstration of intranuclear inclusions in the brain tissue. In case no. 68 the autopsy finding was the presence of an inflammatory lesion in the left occipital lobe. Owing to the fact that the serum specimens obtained from this patient hat not been investigated by the complement fixation test for herpes until a few weeks after death had occurred, no particular attention was paid at autopsy to the possibility of herpetic encephalitis. For the same reason no attempt was made to isolate virus from the brain tissue, nor were intranuclear inclusions especially looked for at the histological examination; on later investigation of the preparations none could be discovered. The clinical picture and the autopsy findings are presented in detail in case report no. 2. Patients nos. 4, 10, 43 and 67 had been admitted to various hospitals with a diagnosis of meningitis serosa. In all these

cases the clinical picture was that of benign aseptic meningitis, and complete recovery took place within a few weeks.

This herpes encephalomeningitis group includes the youngest (15 days, no. 58) and the oldest (63 years, no. 68) patient in the whole encephalomeningitis series; the remaining four patients were 4, 7, 11 and 38 years old. Their age distribution corresponds roughly to that in the entire material.

ANTIBODIES IN PATIENTS WITH KAPOSI'S VARICELLIFORM ERUPTION

Previous Investigations

As mentioned on page 12, Seidenberg, in 1941, isolated the virus of herpes simplex from the vesicle fluid of a patient suffering from Kaposi's varicelliform eruption. In 1944 Wenner (157) also succeeded in isolating this virus from the vesicle fluid of 3 patients; furthermore he demonstrated the presence of neutralizing herpes antibodies in the convalescent-phase sera of 2 of these. Lynch *et al.* (103) reported on 4 patients with Kaposi's eruption, 3 of whom had an obvious history of herpes, whilst herpes virus was isolated from the remaining one (47). In the latter, herpes antibodies had already developed when the virus was isolated from the vesicle fluid. Jaquette, Convey and Pillsburg (83) demonstrated a rise in the level of neutralizing herpes antibody in a 6-month-old patient with Kaposi's eruption, from whom they had isolated the virus on a rabbit's cornea. Ruchman, Welsh and Dodd (127) isolated herpes virus from 4 patients, 3 of whom were adults, and demonstrated a rise in antibody titre in 2 of these. In a case of Kaposi's varicelliform eruption in a 37-year-old man, Kipping and Downie (89) showed that the virus of herpes simplex was the causative agent by isolating it from vesicle fluid and by serological demonstration of an increase in antibody titres. Ruchman and Dodd (125) presented 5 cases, *i.e.* 3 children and 2 adults. From two children and one adult they were able to isolate herpes virus, and neutralizing antibody to herpes developed in all five during the course of the illness. An increase in complement-fixing herpes antibody was demonstrated by Dudgeon (44) in 4, and by Gajdusek, Robbins and Robbins (61) in 3 patients suffering from Kaposi's eruption. Successful isolation of the virus of herpes simplex was reported by Brain, Dudgeon and Philpott (28) in 3, and by Baker, Lawton and

Mc Carthy (13) in one case of this disease; furthermore an increase in both complement-fixing and neutralizing herpes antibodies was demonstrated in all these cases. Isolation of the virus without demonstration of antibody has been reported by Barker and Hallinger (15), Starck (146), Finberg and Easton (49), and Grist (72). In a series of 9 cases of Kaposi's eruption the latter isolated vaccinia virus in 6 and herpes virus in 3.

Own Investigations

The present series of patients suffering from Kaposi's varicelliform eruption has been reported on in detail by Landtman *et al.* in 1955 (94). Hence only the principal data are presented here.

During the period from December 31, 1953, to March 3, 1954, 3 patients with infantile eczema, suddenly complicated by fever and a vesicular eruption, were admitted to the Children's Clinic of the University of Helsinki. They were all admitted to the same ward, where other eczema patients were simultaneously being treated. The first patient (no. 1) infected the patient with eczema in the neighbouring bed (no. 2), who developed a similar eruption accompanied by fever. The second patient exhibiting Kaposi's eruption on admission (no. 3) was hospitalized about one month later; he infected a total of 4 children (nos. 4, 5, 6, 7) in the ward. On March 3, a third patient (no. 8) with this disease was admitted to the ward.

The age and the herpes complement fixation titres of the patients are presented in Table 12. In patient no. 1, from whom a serum specimen was drawn 13 days after onset of the illness, and in patient no. 2, who had been infected by the former, the level of herpes antibody remained constant. In patient no. 3, and in nos. 5, 6 and 7, who had obviously been infected by no. 3, a definite increase in antibodies was demonstrated during the course of the disease. In case no. 4 antibody was not present at the acute stage, and convalescent-phase serum was not obtained, because this patient died after 10 days' illness. In case no. 8 the specimens showed fixation with the control egg antigen and could not therefore be tested by the complement fixation technique here employed.

These results seem to constitute evidence that herpes virus was the causative factor in case no. 3 at least and obviously in all cases infected by him (nos. 4, 5, 6 and 7).

TABLE 12

RESULTS OF HERPES COMPLEMENT FIXATION TESTS IN PATIENTS WITH KAPOSI'S VARICELLIFORM ERUPTION

Patient No.	Age	Period after onset	Herpes complement fixation titres
1.	4 months	13 days	16[1]
		30 days	16
2.	9 months	1 day	4
		13 days	4
3.	6 months	8 days	8
		17 days	64
		23 days	128
4.	10 months	5 days	<4
5.	8 months	4 days	4
		15 days	8
		19 days	16
6.	11.5 months	1 day	<4
		12 days	32
7.	1.5 months	1 day	<4
		9 days	16
		28 days	4
8.	10 months	3 days	—
		11 days	—

[1] reciprocals of serum dilutions

Attempts were made to isolate virus from patients nos. 5, 6 and 7 without success. The failure might have been due to the fact that the vesicles were very small in these cases and did not contain much fluid. The technique in collecting samples consisted in washing out the vesicles with saline containing 20 per cent broth. It is possible that better results would have been obtained if, instead, a suspension had been made of harvested crusts, or if the exudate from underneath removed crusts had been employed. Furthermore the negative results may have been influenced by the fact that the sample from patient no. 5 was recovered after the acute phase of the illness, and that the patients nos. 6 and 7, from whom samples were collected at an earlier stage, had been given blood transfusions

(no. 6) or gamma globulin (no. 7). On the other hand the herpes antibody present in the blood and gamma globulin may have caused an increase in the complement fixation titres which would, perhaps, explain the sudden drop in the level demonstrable in case no. 7.

ANTIBODIES IN PATIENTS WITH PNEUMONIA

According to clinical experience patients with pneumonia very often develop herpetic vesicles. If it should be found that all, or at least the majority of patients suffering from pneumonia, are carriers of herpes virus, some correlation between these two circumstances might be assumed. In order to elucidate this point, herpes complement fixation tests were carried out with sera from 56 adults suffering from lobar or bronchial pneumonia. The results are presented in Table 13. Herpes antibody was found to be present in 31

TABLE 13.

COMPLEMENT-FIXING HERPES ANTIBODIES IN PATIENTS WITH PNEUMONIA, POLIOMYELITIS AND HERPANGINA

Diagnosis	Total number of sera tested	Number of positive sera	Number of negative sera	% positive
Pneumonia	56	31	25	55
Poliomyelitis	37	14	23	38
Herpangina	12	6	6	50

cases, *viz.* in 55 per cent. In comparison with the incidence in normal adults, which was 45 per cent in conscripts and 43 per cent amongst medical students, there is no significant difference which would warrant any conclusions.

In 14 cases both acute- and convalescent-phase sera were obtained. The complement fixation results on these are presented in Table 8, page 38. In no case where herpes antibody was not present in the acute phase did it develop later. In 11 of these patients, who had herpes antibodies in the acute phase, the titre remained constant in 8, rose in 2 and dropped in 1. The change was twofold in all three cases.

ANTIBODIES IN PATIENTS WITH POLIOMYELITIS

Table 13 also shows the results in the tests for herpes antibody carried out with sera from 37 patients suffering from poliomyelitis. The response was positive in 38 per cent. Since 8 of these patients were under 3 years old, and 10 were 3 to 7 years old, the finding broadly corresponds to the incidence in normal subjects, thus being at variance with Weyer's (158) results in 1932. Using the neutralization test in mice, the latter was not able to demonstrate herpes antibodies in any case in a series of 27 polio patients, 22 of whom were from 0 to 12 years old; in the remaining 5 cases age was not known. On the other hand the present results are in agreement with those of Burnet and Lush (34). Using the chorioallantoic membrane technique, these workers studied neutralizing herpes antibodies in the sera of 14 patients with poliomyelitis and demonstrated antibodies in 8 cases.

In 23 cases both acute- and convalescent-phase sera were obtained. The complement fixation results are listed in Table 8, page 38. In no case where antibodies were lacking in the acute phase did they develop during convalescence. In 10 patients, who had herpes antibodies in the acute phase, the titre remained constant in 7, rose in one and dropped in 2. The change was twofold in all three cases.

ANTIBODIES IN PATIENTS WITH HERPANGINA

Evidence having been produced that herpangina is not invariably caused by the Coxsackie viruses (118), the occurrence of herpes antibodies in patients suffering from this vesicle-producing disease was also studied. Acute-phase sera were available from 12 patients with herpangina, and convalescent-phase sera from 3 of these.[1] The results obtained in complement fixation tests are shown in Table 13. Antibody was present in 50 per cent. Of the 3 patients from whom two specimens were obtained, one had no antibodies on either occasion, whilst in the other two, whose sera were positive, the titre remained constant.

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[1] The sera were kindly placed at the writer's disposal by Dr. Pirkko Pohjanpelto.

ANTIBODIES IN PATIENTS WITH TUBERCULOUS MENINGITIS

In mild cases of tuberculous meningitis and severe cases of aseptic meningitis the clinical picture is sometimes similar at the initial stage of the disease. Differential diagnosis may be difficult,

TABLE 14

RESULTS OF HERPES COMPLEMENT FIXATION TESTS IN PATIENTS WITH TUBERCULOUS MENINGITIS

Patient No.	Age	Period after onset	Herpes complement fixation titres
1	11 years	12 days	<4 [1]
		23 days	<4
2	5 years	21 days	8
		28 days	4
3	20 years	10 days	64
		20 days	64
4	9 years	1.5 months	<4
		2.5 months	<4
5	3 years	1 month	4
		1.5 months	8
		2 months	4
6	3 years	18 days	<4
		29 days	<4
7	5 years	15 days	<4
		1.5 months	256
		2.5 months	128
		3 months	32
8	8 years	8 days	<4
		28 days	<4
9	3 years	10 days	<4
		17 days	<4
10	23 years	5 days	<4
		18 days	<4
11	13 years	9 days	<4
		19 days	<4
		24 days	<4

[1] reciprocals of serum dilutions

in particular if rapid improvement has taken place as a result of anti-tuberculous therapy given, for instance, at a provincial hospital during the initial stage of the illness, before an accurate diagnosis has been possible. When, in such cases, the patient is transferred to a specialized department of a central hospital, the continuation of treatment depends on whether the patient is really suffering from tuberculous meningitis which has reacted favourably to the therapy, or from aseptic meningitis which has improved independently of the latter.

In this country, the treatment of tuberculous meningitis in children is mostly concentrated at the Children's Clinic of the University of Helsinki. In dubious cases serum specimens have sometimes been submitted for serological tests. Complement fixation tests for herpes were only carried out in 11 instances, but in view of the fact that a definite increase in titre was demonstrable in one case, there seems to be reason for including this material here. Age and complement fixation results are shown in Table 14. In addition to the sera obtained from the Children's Clinic, the material includes two adult cases (nos. 3 and 10) of tuberculous meningitis from the Aurora Hospital. The age of the children varied from 3 to 11 years. The herpes antibody level remained largely constant in all but patient no. 7, in whom the complement fixation titre rose from $1/<4$ to 1/256, and subsequently decreased to 1/128 and 1/32. In this case tubercle bacilli were later isolated from the cerebrospinal fluid, and when death occurred after three and a half months' illness, the diagnosis of tuberculous meningitis was confirmed at autopsy (case report no. 3). On account of the serological findings it seems obvious, however, that this patient simultaneously suffered from a primary herpes infection. Since neither stomatitis nor any other manifestations of herpetic disease were observable, a subclinical primary herpes infection, or herpes encephalomeningitis present simultaneously with tuberculous meningitis, may have been involved.

CASE REPORTS

Case 1. — L. V., 4605/52, the Children's Clinic of the University of Helsinki. A 6-year-old girl was admitted on Nov. 30, 1952, with a diagnosis of acute stomatitis. Previously she had been in good health, although prone to infections. She became ill on Nov. 11 with high fever, which sub-

sided after five days' penicillin and terramycin therapy. On Nov. 27 the temperature rose again to 39.7°C, and simultaneously vesicles developed in the mouth and on the lips. This time the patient was given chloromycetin, but as the condition was aggravated the child was transferred to hospital. On admission her temperature was 39.0°C. The tongue, the mucous membrane of the mouth, and the lips were covered with greyish plaques and with ulcers, and there was discharge of mucous material from the mouth. Vesicles were also present around the anus and genitals. The patient was given penicillin and sulpha for two days, but nonetheless the temperature rose to 40°C. This therapy was then changed for 200 mg of aureomycin four times a day. The child was feverish until Dec. 11. Four days following admission small vesicles, resembling petechiae, also developed on the soles and palms.

On Dec. 1, laboratory studies showed 9500 white blood cells per cmm, 80 per cent of which were polymorphonuclears and 12 per cent mononuclears. The Wassermann test was negative. Throat swab cultures yielded *Neisseria catarrhalis*. On Dec. 3 virus was isolated from mucous saliva from the patient's mouth. It produced typical herpetic pocks on the chorioallantoic membrane of embryonated eggs, and on intracerebral injection into mice it caused death of the animals within five days. The infectivity of the isolated strain deteriorated, however, during preservation in a deep-freeze before identification. The herpes complement fixation titres showed a definite rise during the course of the illness (Table 10, patient no. 2). Neutralizing antibodies also developed.

On Dec. 16, when the patient was discharged three weeks after onset of the disease, the lips were still rough, but otherwise she was symptom-free. Her mother could not remember there having been any previous episodes of herpetic vesicles, but subsequently such have occurred from time to time.

Summary: A 6-year-old girl fell ill with high fever and vesicular stomatitis accompanied by the development of vesicles also around the anus and genitals, and on the soles and palms. The patient recovered completely within some three weeks. A virus, which obviously must have been herpes simplex, was isolated from the mouth, and in the serum a rise in complement-fixing and neutralizing herpes antibody level was demonstrated.

Case 2. — V. V., 953/54, the Aurora Hospital, Helsinki. A 63-year-old tram driver was admitted on March 13, 1954, with a diagnosis of headache and chronic polyarthritis. Two years previously he had been treated for polyarthritis for a couple of months at a municipal hospital and until January, 1954, he had received gold therapy. Owing to this illness he had been unable for the last two years to extend his elbows. Apart from the polyarthritis, he had been in good condition. On March 4, he had severe headache and fever of 38.6°C. At the same time there was violent vomiting. The temperature dropped in a few days, but vomiting persisted. In addition the patient had vertigo, his eyes hurt, and vision seemed to be impaired.

On admission the temperature was 37.1°C; the general condition was poor. Slight stiffness of the neck and marked stiffness of the back were observed. Spinal fluid examination revealed: Pandy + +, Nonne +, total protein 1.46‰, and 118 white blood cells per cmm, 82 per cent of which were mononuclears. At the hospital he was again feverish for some days. The general condition improved, however, until on March 17 the patient became unconscious for a couple of days following a lumbar puncture. Simultaneously the stiffness of the neck increased, and speech became more difficult. Vision and memory were also impaired. At this stage the symptoms so much resembled those of tuberculous meningitis that massive antituberculous therapy was instituted. The patient was given dihydrostreptomycin, PAS and isoniazide, and streptomycin also by the intralumbar route. The illness steadily progressed, however, the spinal fluid showing a pressure of 290—250 mmH_2O, Nonne and Pandy +, and some 10 to 20 cells. At the end of March the right hand and leg became paralyzed, and the patient gradually lost consciousness completely. Death occurred on April 17, 44 days after onset of the first symptoms.

At autopsy the most significant findings were made in the brain. The meninges displayed no evident change, but the brain tissue was swollen, and the convolutions were flattened. The brain tissue was throughout oedematous, and the posterior part of the left hemisphere was necrotic. In the occipital lobe an irregular, ill-defined, in part necrotic lesion, resembling glioma, was found; on histological examination, however, no gliomatous tissue was observed; it consisted of granulomatous tissue, showing vascular obliteration, perivascular proliferative reaction, exudate cells, and necrosis.

Summary: A 63-year-old man, who had suffered from polyarthritis for a couple of years, suddenly developed violent headache and slight fever. At the hospital stiffness of the neck and changes in the spinal fluid were observed, chiefly indicative of tuberculous meningitis. The illness steadily progressed, the extremities became paralyzed, and finally the patient became unconscious. Death occurred about one and a half months after onset of the disease. The most significant autopsy finding was the presence of an inflammatory lesion in the left occipital lobe. Complement-fixing herpes antibodies developed in the patient's serum during the illness (Table 12, patient no. 68).

Case 3. — M. A., 847/54, the Children's Clinic of the University of Helsinki. On Feb. 12, 1954, a 5-year-old girl was admitted with a diagnosis of tuberculous meningitis; she was unconscious on admission. The family history included many cases of tuberculosis. In the beginning of January, 1954, there was fatigue, occasional fever, and anorexia. On Jan. 26, the patient complained of severe headache, and the temperature was 41°C. She was transferred to the local hospital, where she was given penicillin and streptomycin. On Feb. 10 the neck was stiff and she was semiconscious.

On admission the spinal fluid contained 500 white blood cells per cmm,

16 per cent of which were polymorphonuclears and 84 per cent mononuclears; Nonne and Pandy were +, and sugar was 0.025 per cent. Examination of the blood revealed 13000 white blood cells, 80 per cent of which were polymorphonuclears and 18.5 per cent mononuclears. Cultivation of tubercle bacilli from the spinal fluid was positive. Therapy with PAS, isoniazide and streptomycin was instituted. A few days following admission consciousness was partially resumed, so that the patient was able to apprehend speech. On Feb. 23 she became completely unconscious, and the illness steadily progressed. On Feb. 26 a trepanation and drainage was performed as a last therapeutic resource. When, on March 3, no visible results had been obtained by treatment, medical therapy was discontinued and the catheter only was left in position. On April 27 the latter was also withdrawn because suppuration had developed. Death occurred on May 13, after three and a half months' illness. The diagnosis of violent tuberculous meningitis was confirmed at autopsy. The development of complement-fixing herpes antibodies is demonstrated in Table 14 (patient no. 7).

DISCUSSION

The study of the age incidence of antibodies seems to render valuable information with regard to the virus of herpes simplex, considering that the clinical conditions due to it vary, and in view of the fact that the latter are mostly so benign and non-epidemic that they are not included in the official records; hence their occurrence cannot be studied from any statistical data available. Another factor contributing to the usefulnes of such a study is that herpes simplex antibodies obviously remain in the serum of individuals who have once suffered from a primary infection.

The results of the present investigation into the age incidence of herpetic antibodies seem to indicate that herpes simplex infections during the first year of life are very rare, although at least the complement-fixing herpes antibodies passively transferred from the mother during pregnancy disappear from the serum of the infant by the third month after birth. It might be assumed that the resistance of children under one year old is due to the presence of neutralizing herpes antibodies in the serum after the complement-fixing antibodies have disappeared. Evidence that the former persist for about seven months was, in fact, produced by Anderson and Hamilton (8), but their results, too, revealed an interval of about seven months between the disappearance of antibodies and the onset of the first infection. They referred this resis-

tance to herpetic infection of children under one year old to genetical or to immunological factors, and suggested that a survey of the age incidence of herpetic stomatitis in children of non-herpetic mothers would throw light on this point. Considering that nearly fifty per cent of the infants included in the present material had no antibodies passively transferred from the mother, and that they did not suffer from any herpetic infection at least during their first 12 months of life, it may be concluded that factors other than immunological are also responsible for the absence of herpetic infection in children under one year old. Another interesting aspect of this problem is that children under one who lack herpes antibodies are very prone to herpetic infection if they have skin lesions, as was found in the present investigation of patients suffering from Kaposi's varicelliform eruption. The resistance to herpes virus in childhood seems to be closely related to the question of why a proportion of the population never during their life-time become infected with herpes virus. Is this due to the absence of contact with the virus, or is some constitutional, perhaps hereditary, factor involved which prevents the development of herpetic disease? In the latter case it would seem a reasonable assumption that the children of non-herpetic mothers, who are born without herpes antibodies, are resistant to infection with the virus of herpes during their first year of life and continue to be so, whilst in the children of herpetic mothers, the antibodies passively transferred persist for at least 12 months, even if they are not »circulating». This point might be elucidated by repeatedly testing children of non-herpetic mothers for herpes antibody over a long period.

Acute infectious gingivostomatitis has been regarded as a primary herpes simplex infection (8, 22, 31, 34, 35, 41, 69, 122, 136). Even though the present series of acute aphthous and vesicular stomatitis is small and unselected with regard to clinical picture and age, it is remarkable that a rise in antibody titre was not observed during the course of illness in more than one-third or one-quarter of cases. This may in part be referred to the fact that in many cases the convalescent-phase serum was collected some two weeks after onset, which may be too early; thus an increase in antibody may in these cases have escaped attention. However, the series includes patients whose sera did not show herpes antibodies although specimens were collected many weeks after onset of the

disease. Hence it seems that etiological factors other than the virus of herpes simplex are sometimes the cause of aphthous and vesicular stomatitis even in children. In previous investigations of more extensive series, in which the increase in herpes antibody has been followed serologically, only Anderson and Hamilton (cf. p. 49) observed an increase in herpes antibody titre in all patients with stomatitis. With regard to their series it should be recalled, however, that it included only children from a residential institution (an orphanage), where herpes stomatitis always occurred as an epidemic on a limited scale; hence their results cannot be generalized. In any case the proportion of herpetic stomatitis in the present series of stomatitis is markedly lower than the figures previously reported.

In the encephalomeningitis series a definite increase in herpes simplex antibody was demonstrable in about 8 per cent of cases. This tallies more or less with Afzelius-Alm's (4) corresponding percentage figure, but it is higher than that of Adair, Gauld and Smadel (3). In addition to the six cases showing a definite rise in titre, a twofold increase was demonstrable in six other cases, where the first specimen showed antibodies. It may be assumed that some of these patients suffered from a severe recurrent herpes infection accompanied by slight symptoms of meningitis.

In a patient with tuberculous meningitis, in whom a trepanation and drainage was performed, a marked increase in herpes simplex antibody was demonstrated during the course of the illness. Since neither stomatitis nor any other symptoms of primary herpes infection were present, it is possible that she had a subclinical herpes infection; another possible explanation is that the brain tissue was infected with herpes virus via the catheter, and that the symptoms of herpetic encephalitis were masked by the manifestations of fatal tuberculous meningitis. Such infection with virus seems in any case to be a theoretical possibility, since patients with catheters often incur bacterial infection via the latter in spite of prophylactic antibiotic therapy.

SUMMARY

1) Employing combined amniotic-allantoic fluid antigen, a herpes complement fixation test was elaborated, the usefulness of which in the investigation of herpes simplex antibodies in human sera was proved under adequately controlled experimental conditions.

2) It was found that different strains of the virus of herpes simplex possess a varying capacity to produce complement-fixing antigen in the embryonated hen's egg, particularly in the extra-embryonic fluid; hence the success of the test under investigation was found to depend on the choice of a suitable strain.

The combined amniotic-allantoic fluid antigen was found to be superior to antigen prepared from the chorioallantoic membrane, because the extra-embryonic fluid did not possess anti-complementary properties such as were found with membrane suspension. Furthermore the yield per egg of amniotic-allantoic fluid antigen was larger than the yield of membrane antigen.

A long fixation time (20 hours at 4°C) was also found to be essential for successful tests.

3) In a selected series of sera from 31 patients, no correlation was demonstrable between the herpes complement fixation titres and the mumps complement fixation titres, which were also obtained with extra-embryonic fluid antigen.

In neutralization tests on the chorioallantoic membrane, 10 out of 30 unselected sera were found to contain herpes simplex antibodies. The same 10 sera were the only ones in the series showing herpes antibodies in the complement fixation test.

In a group of 21 medical students, who gave a positive history of herpes vesicles, all had complement-fixing herpes antibodies.

Acute- and convalescent-phase serum specimens collected from 45 patients with pneumonia, poliomyelitis and mumps did not display more than a twofold change in herpes complement fixation titre during the course of the illness.

4) A series of 599 sera representing different age groups was investigated for herpes antibody using the complement fixation test. The incidence was as follows: 57 per cent in cord sera and about the same in the group 0 to 3 months; 2 per cent in the group 3 to 12 months; 25 per cent in the group 1 to 3 years; 38 per cent in the group 3 to 7 years, and 46 per cent in the group 7 to 15 years. The last-mentioned figure broadly corresponds to the incidence of antibody noted in adults.

5) Herpes antibodies were investigated in 28 patients with acute aphthous or vesicular stomatitis, the majority of whom were children. Acute- and convalescent-phase sera were obtained in 12 cases, 3 of which showed a definite rise in herpes antibody titre. Furthermore a twofold increase was demonstrated in 2 cases, in which the two serum specimens were collected at an interval of only a few days.

6) Herpes antibodies were investigated in 91 sporadic cases of acute encephalomeningitis. Out of 78 of these, who constituted a selected series, 6, or about 8 per cent, showed an increase by fourfold or more in herpes antibody titre. Furthermore a twofold increase was demonstrable in 6 patients.

7) In the first of 3 patients with Kaposi's varicelliform eruption who were admitted to a children's hospital, the herpes complement fixation titre remained unchanged during the course of the illness, and the same was found in another patient who had been infected by the former. In the second of the above-mentioned three patients the titre rose from 1/8 to 1/128. Out of 4 patients infected by him, 3 showed an increase in antibody (one died). The serum of the third patient who had Kaposi's eruption on admission could not be tested for herpes antibody by the complement fixation technique employed.

8) Herpes antibodies were present in 55 per cent of 56 patients with pneumonia, in 38 per cent of 37 patients with poliomyelitis, and in 50 per cent of 12 patients with herpangina. In no case of pneumonia, poliomyelitis or herpangina where herpes antibodies were absent in the acute phase of the disease were they demonstrable in convalescent-phase serum.

9) In a series of 11 patients with tuberculous meningitis the herpes antibody titre rose from $1/<4$ to 1/256 in one fatal case, where a trepanation and drainage was performed.

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